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The reduction and induction of Kaposi's sarcoma-associated herpesvirus lytic replication

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Abstract

In humans, Kaposi's Sarcoma-associated Herpesvirus (KSHV) is capable of establishing latent infection in B-cells. During latency no infectious virions are produced, the viral episome is maintained and few viral genes are expressed. Latently infected cells, however, retain the capacity to enter the lytic cycle. KSHV ORF50 encodes the transcription factor RTA whose expression is sufficient to initiate the full lytic cycle.

RNA interference (RNAi) involves the sequence specific silencing of gene expression. Although extensively validated and widely available at present, lentiviral vector-mediated RNAi was unavailable in 2002. This thesis describes the generation and characterisation of shRNA expression cassettes that can be delivered using self-inactivating lentiviral vectors. Using shRNAs designed to interfere with KSHV ORF50 expression, the number of cells expressing RTA following chemical induction of the lytic cycle was greatly reduced. Analysis of viral lytic antigens and virus production suggests that reducing RTA expression prevents latent KSHV from initiating the lytic cycle.

Although KSHV infects B-cells *in vivo*, most B-cell lines are recalcitrant to KSHV infection *in vitro*. This thesis describes the *in vitro* susceptibility of a panel of B-cell lines, representing different stages of B-cell differentiation, and other common cell-lines to recombinant KSHV infection. Interestingly, all the adherent cell-lines examined were susceptible to recombinant KSHV infection whereas we were unable to identify any B-cell lines which were efficient targets for recombinant KSHV infection

KSHV positive primary effusion lymphoma (PEL) cells are phenotypically similar to plasmablasts, which represent a late stage in B-cell differentiation, immediately preceding terminal differentiation into plasma cells. This thesis examines the stage at which B-cell development is arrested in PEL, focussing on the transcription factor X-box binding protein-1 (XBP-1) and the unfolded protein response (UPR). Using exogenously expressed XBP-1, the role of XBP-1 as the possible molecular switch linking terminal differentiation and KSHV lytic reactivation is considered.

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Contents

Abbreviations

(Ψ)	Packaging sequence
181GB1-4	Transformed human brain endothelial cells
Activated-DLBCL	Activated B-like diffuse large B-cell lymphoma
Ag	Antigen
Ago	Argonaute
AID	Activation induced deaminase
AIDS	Acquired immunodeficiency syndrome
AIDS-KS	AIDS-associated KS
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
AP-1	Activating protein 1
APC	Antigen presenting cell
ATCC	American Type Culture Collection
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BAFF	B-cell activating factor of the tumour-necrosis-factor family
BART	Bam A rightward transcript
BB19	Brain-derived endothelial cell-line
BCBL	Body cavity-based lymphoma
BCL	B-cell lymphoma
BCR	B-cell receptor
BEC	Blood vessel endothelial cell
BHK-21	Baby hamster kidney cells
BL	Burkitt's lymphoma
BLIMP-1	B-lymphocyte-induced maturation protein-1
bp	Base-pair
BSAP	B-cell specific activator protein
bZIP	Basic leucine zipper
C/EBPα	CCAAT/enhancer binding protein alpha
CBP	CRE-binding protein
CHELI	Chediak-Higachi syndrome cells
CHO	Chinese hamster ovary
CLL	Chronic lymphocytic leukaemia
cPPT	Central polypurine tract
CRE	Cyclic AMP response element
CSDW	cPPT-SFFV-dEGFP-WPRE
CSGW	cPPT-SFFV-EmGFP-WPRE
CSPW	cPPT-SFFV-PAC-WPRE
CSR	Class switch recombination
CSRW	cPPT-SFFV-DsRed-WPRE
CV-1	African green monkey kidney cell-line
D	Ig diverse region
DC	Dendritic cell
dEGFP	Destabilised GFP
DGCR8	DiGeorge syndrome critical region gene 8
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco's Modified Eagle Medium
DMVEC	Dermal microvascular endothelial cell
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate mix
dsDNA	Double stranded DNA
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
eIF2α	Eukaryotic initiation factor 2α
EMA	Epithelial membrane antigen
EMCV	Encephalomyocarditis virus

EmGFP	Emerald GFP
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FDCs	Follicular dendritic cell
FL	Follicular lymphoma
FLICE	FADD interleukin-1□-converting enzyme
FLIP	FLICE inhibitory protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Interferon-□ activation site
GC	Germinal centre
GFP	Green fluorescent protein
HAT	Histone acetyl transferase
HCF	Host-cell factor
HCL	Hairy cell leukaemia
HCMV	Human cytomegalovirus
HFF	Human foreskin fibroblasts
HHV	Human-herpesvirus
HIF	Hypoxia inducible factor
HIV-1	Human immunodeficiency virus-1
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
HSV	Herpes-simplex virus
HUVEC	Human umbilical vein endothelial cell
HVS	Herpesvirus saimiri
Ig	Immunoglobulin
IgH	Ig heavy chain
IgL	Ig light chain
IKKβ	Inhibitor of kappa B kinase beta
IL	Interleukin
IPK	Inhibitor of PKR
IPTG	Isopropylthio-β-D-galactoside
IRE1	High inositol-requiring protein 1
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF-3γ	Interferon stimulated gene factor 3 gamma
ISRE	Interferon-stimulated response element
IU	Infectious units
J	Joining
K15 M	K15 minor form
K15 P	K15 predominant form
kb	Kilobase
KDR	Kinase domain receptor
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
KV-1	KSHV deletion mutant
LANA	Latent nuclear antigen
LANA-2	Latency-associated nuclear antigen-2
LAT	Latency-associated transcript
LB	Luria-Bertani
LCL	Lymphoblastoid cell line
LEC	Lymphatic endothelial cell
LMP	Latent membrane protein
Ln-Cap	Prostate cancer derived cell line
Log(2)	Log base 2
LTR	Long terminal repeat
LUR	Long unique region
MAPK	Mitogen-activated protein kinase
MCD	Multicentric Castleman's disease
MCL	Mantle cell lymphoma
Mcl-1	Myeloid cell leukaemia-1

MeCP	Methyl CPG binding proteins
MFI	Mean fluorescence intensity
MHV-68	Murine gammaherpesvirus 68
miRISC	Micro RNA induced silencing complex
miRNA	Micro RNA
MITF	Micropthalmia-associated transcription factor
MM	Multiple myeloma
MOI	Multiplicity of infection
MTA3	Metastasis-associated 1 family member 3
MZ	Marginal zone
NCI	National Cancer Institute
NFκB	Nuclear factor kappa B
NPC	Nuclear pore complex
NRF2	Nuclear factor erythroid 2 (NF-E2)-related factor 2
Nut-1	Nuclear transcript 1
OAS	2'-5' oligoadenylate synthase
OMK637	Owl monkey kidney cells
ORF	Open reading frame
Ori-Lyt	Origin of lytic replication
P/S	Penicillin and streptomycin
PAA	Phosphonoacetic acid
PALS	Periarteriolar lymphoid sheath
PAN	Polyadenylated nuclear transcript
PAZ	Piwi/Argonaute/zwille
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCL	Plasma cell leukaemia
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PERK	PKR-like ER kinase
PF-8	Processivity factor 8
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKR	Double-stranded DNA dependent protein kinase
pre-B-cells	Precursor B-cell
Pre-BCR	Pre-B-cell receptor complex
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
Pro-B-cells	Progenitor B-cell
PTLD	Post-transplant lymphoproliferative disorder
QT-6	Quail fibroblast cell line
RAG	Recombinase-activating gene
rasiRNAs	Repeat associated siRNA
RBP-Jκ	Recombination-signal binding protein Ig J□ region
RDA	Representational difference analysis
RDRP	RNA-dependent RNA polymerase
RFHVMn	Retroperitoneal fibromatosis-associated herpesvirus *Macacca nemestrina*
RIG-I	Retinoic acid inducible gene 1
RISC	RNA induced silencing complex
RITS	RNA induced transcriptional silencing complex
RLC	RISC loading complex
RMEC-1	Mesoendothelial cells
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
RRE	Rev response element
RT	Reverse transcriptase
RTA	Regulator of transcription activation
S1P	Site 1 protease
S2P	Site 2 protease

SAIDS	Simian AIDS
SCC15	Squamous cell carcinoma of the tongue
SCF	Stem-cell factor
SDF-1	Stromal cell-derived factor-1
shRNA	Short hairpin RNA
SIN	Self-inactivating
siRNA	Small/short interfering RNAs
SLC	Surrogate light chain
SOX	Shutoff and exonuclease
SP-1	Specificity protein 1
SSB	ssDNA binding protein
ssDNA	Single stranded DNA
STAT	Signal transducer and activator of transcription
T 1.1	Transcript 1.1
TAE	Tris-acetate-EDTA
TCR	T-cell receptor
TdT	Terminal deoxynucleotide transferase
TE	Tris-EDTA
T_H	Helper T-cell
TK	Thymidine kinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPA	12-0-tetradecoylphorbol 13-acetate
TR	Terminal repeats
TRAF	Tumour necrosis factor receptor-associated factor
TRBP	Transactivating response RNA-binding protein
TRITC	Tetramethylrhodamine isothiocyanate
tRNA	Transfer RNA
U5	Unique 5'
UCMC	Human umbilical cord mononuclear cells
UPR	Unfolded protein response
UV	Ultra-violet
V	Ig variable region
vBcl-2	Viral Bcl-2
vCyclin	Viral cyclin
VEGF	Vascular endothelial growth factor
vFLIP	Viral FLICE inhibitory protein
vGCPR	Viral G-protein coupled receptor
vIL-6	Viral IL-6
vIRF	Viral interferon regulatory factor
vMIP	Viral macrophage inflammatory protein
vOX-2	Viral OX-2
VP	Virion protein
VSV	Vesicular stomatitis virus
VZV	Varicella-Zoster virus
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
XBP-1	X-box binding protein-1
X-Gal	5-bromo-4-chloro-3-indolyl-□-D-galactoside

Chapter 1

Introduction

1. Kaposi's Sarcoma-associated Herpesvirus (KSHV)

1.1.1. The history of KSHV

In 1872 the Hungarian dermatologist Mauritz Kaposi described an idiopathic multiple pigmented sarcoma of the skin (Kaposi, 1872). This rare angiomatous neoplasm affected elderly men of a Mediterranean, Middle Eastern and African origin and is now described as classic Kaposi's sarcoma (reviewed in Schwartz, 2004). Herpesvirus particles were first described in tissue culture explants of classic Kaposi's sarcoma (KS) in 1972 (Giraldo *et al.*, 1972) although these were thought to be human cytomegalovirus particles (Giraldo *et al.*, 1980). In 1981, during the early stages of the AIDS epidemic, a more aggressive and disseminated form of KS was reported in young homosexual men (Hymes *et al.*, 1981). The predominant risk factor for developing KS was determined to be homosexual sex between men, suggesting an infectious aetiology of AIDS associated KS (Weiss and Biggar, 1986).

Chang and colleagues discovered Kaposi's sarcoma-associated herpesvirus (KSHV) in 1994 through representational difference analysis (Lisitsyn *et al.*, 1993) of AIDS associated KS and adjacent normal skin (Chang *et al.*, 1994). This identified sequences homologous to, but distinct from, capsid and tegument genes of herpesvirus saimiri and Epstein Barr virus indicating an eighth human herpesvirus (HHV-8) had been discovered. KSHV was later classified as a gamma-2-herpesvirus, genus *Rhadinovirus*, the first member of this genus known to infect humans (Moore *et al.*, 1996b). Consistent with the lymphotropic nature of homologous viruses, KSHV sequences were rapidly detected in primary effusion lymphoma cells (Cesarman *et al.*, 1995a). This allowed the propagation of KSHV positive cell-lines (Cesarman *et al.*, 1995b) and the cloning and sequencing of the viral genome (Russo *et al.*, 1996).

KSHV is now widely accepted to be the aetiological agent of Kaposi's sarcoma, the most common malignancy in regions of sub-Saharan Africa (Dedicoat and Newton, 2003). KSHV is also associated with two B-cell neoplasms: primary effusion

lymphoma (Cesarman *et al.*, 1995a) and the plasma cell variant of multicentric Castleman's disease (Soulier *et al.*, 1995).

1.1.2. Human Herpesviruses

KSHV is well adapted to its host and likely coevolved with humans (reviewed in Hayward, 1999). Consistent with this ancient relationship between members of the *Herpesviridae* and *Homo sapiens* the term herpes was used by Hippocrates over 2000 years ago to describe a rash that appeared to creep along the skin. Derived from *herpein,* meaning to creep, the term herpes was used for centuries to describe a variety of skin lesions not necessarily associated with herpesviruses. In 1924 herpesvirology was born when herpes simplex virus (HSV) was isolated and serially transmitted between rabbits (Gruter, 1924).

At present, over 100 herpesviruses have been identified and membership is defined by virion morphology and nucleic acid content (www.ncbi.nlm.nih.gov/ICTVdb). All herpesviruses, by definition, are enveloped viruses that contain an icosahedral particle and double stranded DNA. Herpesviruses can be further subdivided into α, β and γ-herpesviruses. Classification was initially based on host range and properties *in vitro* (Roizman *et al.*, 1981), and subsequently according to genomic DNA sequence. The 8 human herpesviruses are described in table 1.1.2.

Table 1.1.2. Human herpesviruses and their disease associations.

Sub-family	Human herpesvirus	Common name	Disease associations
α	HHV-1	Herpes-simplex virus (HSV)-1	Oropharangeal herpes (cold sores) Genital herpes
α	HHV-2	Herpes-simplex virus (HSV)-2	Genital herpes
α	HHV-3	Varicella-Zoster virus (VZV)	Varicella (chickenpox) Zoster (shingles)
γ	HHV-4	Epstein-Barr virus (EBV)	Infectious mononucleosis Nasopharyngeal carcinoma Burkitt's lymphoma Classical Hodgkin's lymphoma
β	HHV-5	Human cytomegalovirus (HCMV)	CMV-mononucleosis CMV disease
β	HHV-6a HHV-6b		Exanthem subitum (sixth disease) Roseola infantum Encephalitis
β	HHV-7		Exanthem subitum (sixth disease) Encephalitis
γ	HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV)	Kaposi's sarcoma Primary effusion lymphoma Multicentric Castleman's disease

KSHV is a γ-herpesvirus whose closest human homologue is Epstein Barr virus (EBV). The *Gammaherpesvirinae* are defined by their limited host range, lymphocyte tropism and frequent latent infection of lymphoid tissue.

1.1.2.1. The Rhadinoviridae

KSHV is a Rhadinovirus (Moore *et al.*, 1996b) and prior to the discovery of KSHV, herpesvirus saimiri was the archetypal Rhadinovirus (reviewed in Fickenscher and Fleckenstein 2001). Herpesvirus saimiri (HVS) and the closely related herpesvirus ateles infect New World squirrel monkeys (*Saimiri sciureus*) and spider monkeys (*Ateles geoffryi*) respectively. Both are T-lymphotropic viruses capable of causing T-cell lymphoma in numerous non-natural primate hosts (reviewed in Ensser and Fleckenstein, 2005). Rhadinoviruses have also been identified in African green monkeys (Greensill *et al.*, 2000b) and macaques (Desrosiers *et al.*, 1997). In macaques the rhadinoviruses retroperitoneal fibromatosis-associated herpesvirus *Macacca nemestrina* (RFHVMn) and retroperitoneal fibromatosis-associated herpesvirus *Macacca mulatta* (RFHVMm) can cause a proliferation of spindle cells reminiscent of KS (Rose *et al.*, 1997). Interestingly, similar to the association of KS and AIDS, retroperitoneal fibramatosis is associated with simian acquired immunodeficiency syndrome (SAIDS). Rhadinoviruses have also been identified in Old World primates such as Chimpanzees (Greensill *et al.*, 2000a) and Gorillas (Lacoste *et al.*, 2000) although, currently, they are only loosely associated with tumourigenesis.

1.1.3. The KSHV virion

The herpesvirus virion is composed of four morphologically distinct substructures. An inner core of toroidal packaged DNA (Furlong *et al.*, 1972) is surrounded by a structurally repetitive icosahedral capsid. A dense protein layer termed the tegument surrounds the nucleocapsid, which is less structured than the capsid but partially mirrors its symmetry (Zhou *et al.*, 1999; Chen *et al.*, 1999). The mature virion is enveloped in a cellular membrane, enriched with viral glycoproteins, derived from the trans-Golgi network (Skepper *et al.*, 2001).

The KSHV capsid structure has been solved by cryo-electron microscopy. The capsid shell is composed of 162 capsomers arranged in a T=16 icosahedral arrangement (Wu *et al.*, 2000). Recently the composition of KSHV virions have been analysed identifying many host proteins, viral proteins (Bechtel *et al.*, 2005b; Zhu *et al.*, 2005) and mRNAs (Bechtel *et al.*, 2005a) present in the virion. The viral components of the KSHV virion

Table 1.1.3. KSHV virion composition (adapted from Bechtel *et al.*, 2005a; 2005b; Zhu *et al.*, 2005).

Gene	Protein	Protein present in virions?	mRNA present in virions?	Function
ORF17.5	VP 17.5	Yes		Capsid
ORF25	Major capsid protein	Yes		Capsid
ORF26	Minor capsid protein	Yes		Capsid
ORF62	TRI-1	Yes		Assembly/DNA maturation
ORF65	SCIP	Yes		Capsomer-interacting protein
ORF8	gB	Yes		Envelope glycoprotein
ORF K8.1	K 8.1	Yes	Yes	Envelope glycoprotein
ORF22	gH	Yes		Envelope glycoprotein
ORF28		Yes		Envelope
ORF39	gM	Yes		Envelope glycoprotein
ORF47	gL	Yes		Envelope glycoprotein
ORF53	gN	Yes		Envelope glycoprotein
ORF68		Yes		Envelope glycoprotein
ORF21	TK	Yes		Tegument
ORF33		Yes		Tegument
ORF45		Yes		Tegument
ORF63		Yes		Tegument
ORF64		Yes		Tegument
ORF75		Yes		Tegument
ORF6	SSB	Yes		ssDNA binding protein
ORF7		Yes		
ORF11		Yes		
ORF27		Yes		
ORF52		Yes		
ORF50	RTA	Yes		Lytic transactivator
ORF K8	K-bZIP	Yes		Transcription/DNA replication
ORF37	SOX	Yes		Exonuclease-Host shut-off
ORFK2	vIL-6		Yes	Autocrine IL-6 like activity
ORFK4	vMIP-II		Yes	CCR4 agonist
ORFK5	K5		Yes	Downregulate MHC class I
ORFK6	VMIP-I		Yes	CCR8 agonist
ORFK7	K7		Yes	Protein degradation/antiapoptotic
ORF17	Pr and AP		Yes	Protease and assembly protein
ORF54	dUTPase		Yes	
ORF58			Yes	
ORF59	PF-8		Yes	DNA replication
ORF K12	Kaposin A		Yes	Oncogenic/encodes miRNAs

identified in these studies is summarised in table 1.1.3. This list is not comprehensive as most components were identified through mass spectrometry. The regulator of transcription activation (RTA), KSHV basic leucine zipper containing protein (K-bZIP) and shutoff and exonuclease (SOX) protein were identified using specific antibodies (Bechtel *et al.*, 2005b) but their peptides were not detected by mass spectrometry (Bechtel *et al.*, 2005b; Zhu *et al.*, 2005). This suggests that many other unidentified proteins may be present at lower abundance within the virion.

Many host proteins associated with KSHV virions were identified in the studies. Most of these are abundant proteins, such as β-actin and heat shock protein 90, and it is not clear whether they are selectively or randomly incorporated into the virion.

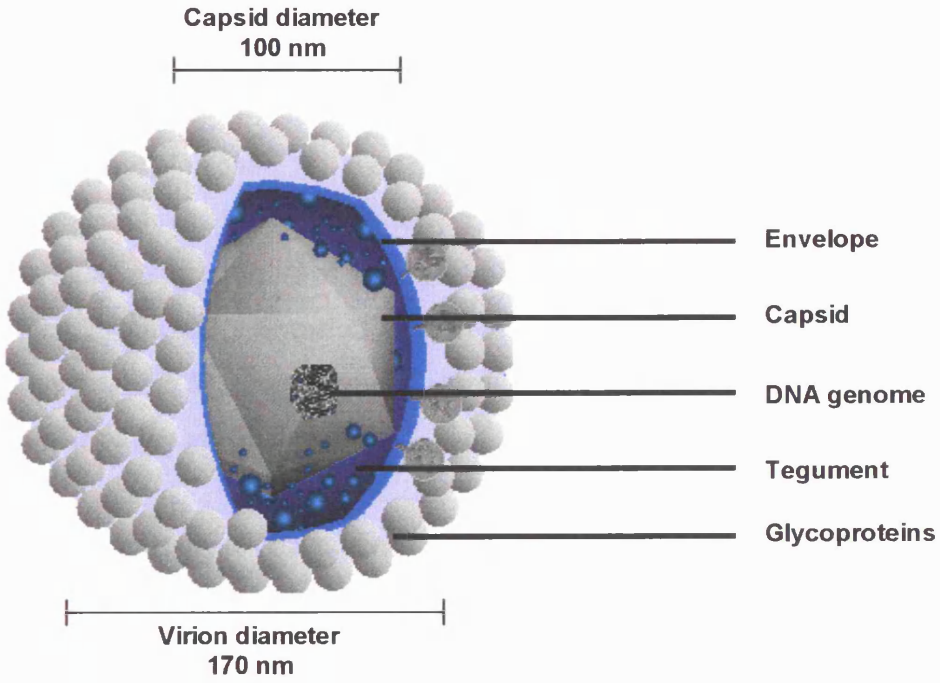


Figure 1.1.3. The KSHV virion
A diagrammatic representation of the KSHV virion is shown. Capsid and virion diameters were determined by Wu and colleagues (Wu *et al.*, 2000) and Wang and Montalvo (Wang *et al.*, 1998) respectively.

1.1.4. The KSHV genome

The KSHV genome exists as a circular dsDNA episome within infected cell nuclei and as linear dsDNA within the virion and during lytic replication. Sequencing of the KSHV genome identified a ~140.5 kilobase (kb) long unique region (LUR) flanked by

multiple 801bp terminal repeat (TR) units (Russo *et al.*, 1996). The repetitive nature of the TR region makes sequence data difficult to interpret making accurate measurements of genome length difficult. The KSHV linear virion-associated genome is estimated to be ~165 kb long (Zhong *et al.*, 1996; Renne *et al.*, 1996a)

The KSHV genome shares the 7-block organisation of other characterised herpesviruses (reviewed in Chee and Barrell, 1990) with gammaherpesvirus, Rhadinovirus and KSHV-specific genes present between blocks (Russo *et al.*, 1996). 81 open reading frames (ORFs) of >100 amino acids were initially assigned to the KSHV LUR. Of these, 66 have homologues in HVS and the KSHV LUR shares almost complete syntenny to HVS. Accordingly, KSHV ORFs were assigned based on HVS nomenclature with ORFs not homologous to HVS genes numbered in consecutive order and prefixed with a K (K1-K15). Since initial characterisation several additional ORFs have been characterised notably K4.1, K4.2, K8.1, K10.1, K10.5, K11.1, K14.1 and many spliced isoforms have been reported (Reviewed in Zheng, 2003).

Rhadinoviruses encode a greater number of cellular homologues than other herpesviruses. KSHV seems to have evolved to encode cellular proteins rather then induce their expression. For example, EBV latent membrane protein 2 is capable of inducing cyclin D2 expression in B-cells (Arvanitakis *et al.*, 1995) whereas KSHV encodes a cyclin D2 homologue (Cesarman *et al.*, 1996). The reasons for this direct molecular piracy are unclear.

Recently herpesviruses have been shown to encode microRNAs (Pfeffer *et al.*, 2004). KSHV is thought to encode 10-11 microRNAs (miRNAs) adjacent to other genes expressed during latency *in vitro* (Pfeffer *et al.*, 2005; Cai *et al.*, 2005 and Samols *et al.*, 2005). The functional targets of these miRNAs have yet to be characterised.

1.1.4.1. KSHV genomic variation

As with all biological entities, KSHV exhibits genomic variation. Analysis of ORF K1 variation identified 4 KSHV subtypes differing 15-30% in their K1 amino acid sequence (Zong *et al.*, 1999). Based on ORF K1 variation, KSHV can be divided into subtypes A-D and the presence of ORF K15 M or P alleles further define the KSHV subtype (Zong *et al.*, 2002). KSHV subtypes correlate with geographical and/or ethnic origin of the infected host and probably arose during geographic isolation of host

populations (Hayward, 1999; Zong *et al.*, 2002). Although ORF K1 is highly variable between subtypes (Zong *et al.*, 1999) this genomic locus seems to be highly polymorphic and estimates of overall genomic variation are approximately 1.5% (Zong *et al.*, 1997). As well as variation within the LUR the TR region is also subject to variation. Similar to EBV, episome circularisation is an unpredictable process resulting in a variable number of repeats in each circularisation event (Raab-Traub and Flynn, 1986). This property has proved useful in analysing the clonality of KSHV-associated tumours (Judde *et al.*, 2000).

As well as subtype variations, large-scale variation of the KSHV genome is also possible. Both duplications (Russo *et al.*, 1996) and deletions (Deng *et al.*, 2004) have been reported in primary effusion lymphoma (PEL) cells *in vivo*. The role of aberrant KSHV genomes in KSHV-associated tumours is unclear, but cells infected with lytic replication defective KSHV have been suggested to have enhanced transformation and proliferation potential and could be relevant in tumour progression (Deng *et al.*, 2004).

1.1.5. KSHV cellular infection

The KSHV lifecycle can be considered at many different scales. This thesis will first describe the molecular events of KSHV infection and replication (Summarised in figure 1.1.5) before considering infection and transmission within and between human hosts (section 1.1.6).

Herpesvirus attachment can be divided into two distinct steps (reviewed in Spear and Longnecker 2003; Garner 2003). Binding initially occurs through adsorption to target cells followed by specific receptor engagement and fusion of the viral envelope with the plasma membrane. Similarly to HSV, KSHV first adsorbs to cells through an interaction between heparin sulphate and glycoprotein K8.1 (Akula *et al.*, 2001) or glycoprotein gB (Birkmann *et al.*, 2001). These interactions increase KSHV infectivity presumably through increasing the probability of KSHV interacting with cellular receptors involved in fusion. However, these interactions are not necessary for KSHV entry (Luna *et al.*, 2004).

Following adsorption, KSHV glycoproteins interact with their cognate receptors. KSHV glycoproteins gB, gH, and gL can all mediate membrane fusion *in vitro* (Pertel, 2002) but the interaction of gB with integrin $\alpha 3\beta 1$ is the only characterised receptor

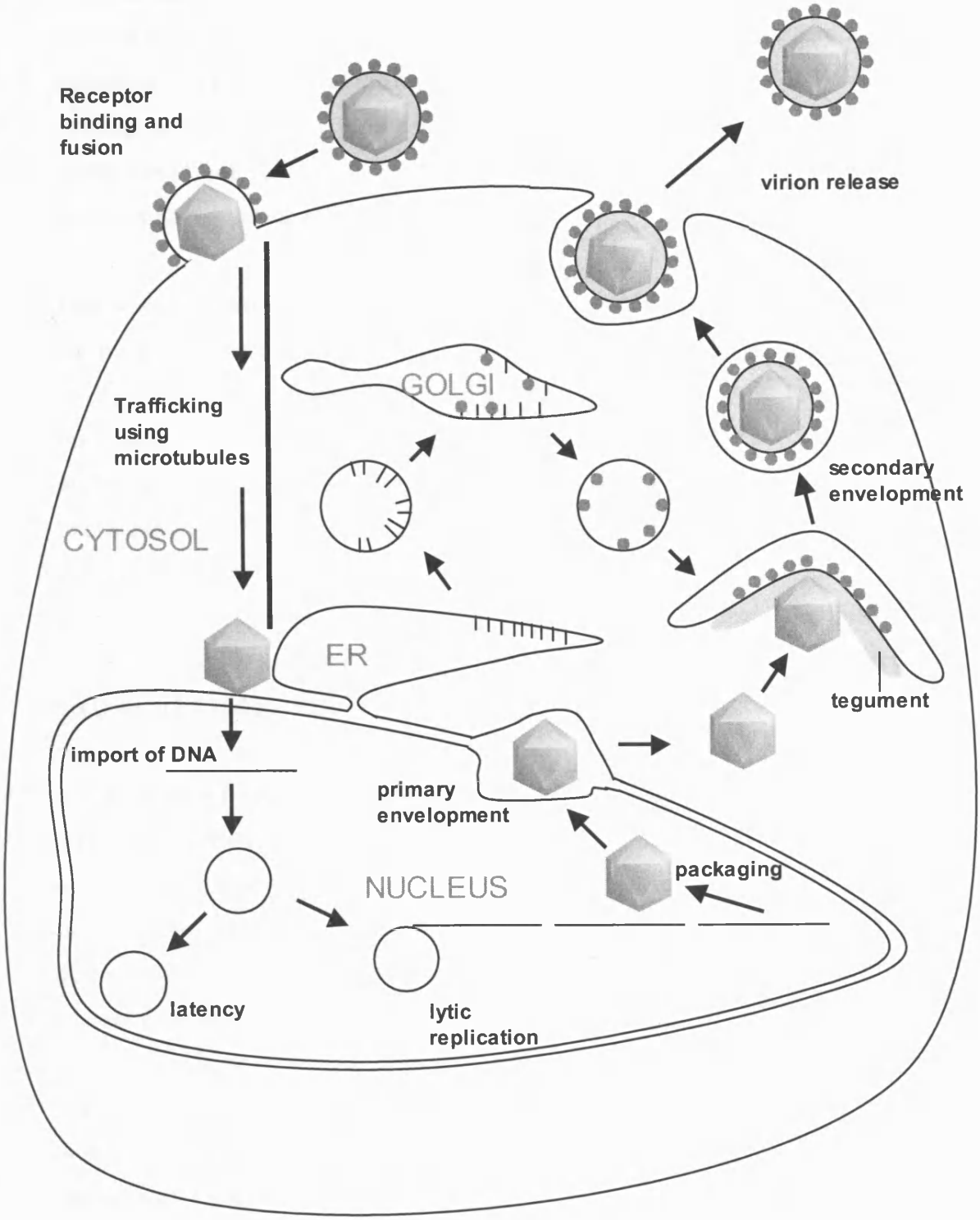


Figure 1.1.5. The replication cycle of human herpesviruses (adapted from Mettenleiter, 2004)
A diagrammatic summary of the herpesvirus lifecycle.

interaction for KSHV (Akula *et al.*, 2002). Although clearly important in KSHV entry, it is not clear whether integrin α3β1 is the main cellular receptor (Inoue *et al.*, 2003). As well as facilitating viral entry, receptor engagement can also initiate intracellular signalling cascades. This is the case for KSHV and integrin α3β1. KSHV engagement of integrin α3β1 activates focal adhesion kinase (Sharma-Walia *et al.*, 2004) a process which could aid penetration of the actin cytoskeleton or even prime cells for the establishment of latency (Sharma-Walia *et al.*, 2005).

Following receptor engagement, membrane fusion ensues. This generally occurs at the plasma membrane, although virion endocytosis prior to fusion has been reported (Akula *et al.*, 2003). After fusion, unenveloped virion particles traffic to the nucleus along microtubules utilising dynein motors (Naranatt *et al.*, 2005). The herpesvirus nucleocapsid is too large to traverse the nuclear pore complex (NPC). Instead, the nucleocapsid is able to dock with the NPC in the presence of importin β and, in an energy dependent manner, is able to eject the herpesvirus genome into the nucleus (reviewed in Greber and Fassati, 2003). Following nuclear entry, the linear genome circularises and the virus enters a lytic or latent replication programme (discussed in sections 1.1.6 and 1.1.11).

1.1.6. KSHV lytic gene expression

Like other herpesviruses, KSHV lytic replication involves viral DNA replication by virus-encoded machinery, and packaging of viral DNA into infectious virions. This is achieved through a tightly regulated gene expression cascade (reviewed in Roizman and Knipe, 2001). Genes expressed during lytic replication can be assigned to different classes depending on how their expression is affected by the protein synthesis inhibitor cycloheximide or viral DNA replication inhibitors such as phosphonoacetic acid. The classification of herpesvirus genes is summarised in table 1.1.6. It is important to remember when considering KSHV gene expression that this classification is an intellectual creation and that designation is not absolute. The complex host-virus relationship means that viral genes respond to the changing host-cell environment as well as viral replication programmes. An example of this is KSHV latent nuclear antigen 2 (LANA2). LANA2 is expressed during latency in B-cells but is exclusively a lytic gene in KS lesions (Rivas *et al.*, 2001). The cell-type and context dependence of this classification system means that single-gene assays may not be reliable at identifying cells harbouring KSHV in the lytic cycle (Krishnan *et al.*, 2004). Rather it

is likely that multiple layers of evidence, in terms of viral gene expression, are required to distinguish latent and lytic KSHV gene expression programmes.

Table 1.1.6. Regulatory classes of herpesvirus genes expressed during lytic replication after *de novo* infection.

Class	Symbol	Definition
Immediate-early	α	Expression requires no prior viral protein synthesis (transcription insensitive to cycloheximide).
Early	β	Expression is independent of virus DNA synthesis (transcription prevented by cycloheximide but insensitive to DNA synthesis inhibitors).
Partial-late	γ1	Expression is increased by viral DNA synthesis (transcription prevented by cycloheximide and reduced by DNA synthesis inhibitors.
True-late	γ2	Expression requires viral DNA synthesis (transcription blocked by cycloheximide or DNA synthesis inhibitors).

Definitions reproduced from Roizman and Knipe (2001).

KSHV RTA expression is necessary and, in most circumstances, sufficient for KSHV lytic replication (Lukac *et al.*, 1998). RTA is an immediate early gene and is a transcriptional activator capable of initiating the lytic cycle (reviewed in West and Wood, 2003). Although several studies have began categorising lytic gene expression (Zhu et al., 1999; Lu *et al.*, 2004) a comprehensive study involving cycloheximide and DNA synthesis inhibitors in the same cell-line has yet to emerge. However, by analogy to HSV lytic replication, immediate early genes tend to encode transcription factors required for early gene expression, early genes tend to be involved in viral DNA replication and late genes tend to be structural, required for the formation of new virions (reviewed in Roizman and Knipe, 2001; Jenner *et al.*, 2001).

1.1.6.1. Abortive lytic replication

Initiation of the KSHV lytic replication cycle does not always ensure faithful completion of that cycle. Partial lytic cycles are termed abortive lytic replication. Although the name insinuates that the lytic cycle has gone wrong, gene expression profiles classified as 'abortive lytic' probably represent tightly regulated but poorly characterised stages of the KSHV lifecycle. The best-characterised abortive lytic expression programme occurs immediately following cellular infection with KSHV.

KSHV virions contain RTA, probably as a component of the tegument (Bechtel *et al.*, 2005b). Following infection this results in initiation of an abortive lytic gene expression programme (Krishnan *et al.*, 2004), followed by supervention of a latency programme. This is similar to HSV-1 whose virions contain virion protein 16 (VP16). VP16 enhances α gene expression following infection, initiating lytic replication (Campbell *et al.*, 1984; Post *et al.*, 1981). It seems that KSHV has modified this conserved herpesvirus mechanism of entering lytic replication immediately following infection to prime cells for latency, the default programme of KSHV infection *in vitro* (Bechtel *et al.*, 2003).

Abortive lytic replication is widely regarded to be necessary for sarcomagenesis (Reviewed in Hayward, 2003; Boshoff, 1998a). These models centre on the involvement of the KSHV viral G-protein coupled receptor (vGPCR) in paracrine stimulation of endothelial cell proliferation. The vGPCR is a lytic gene whose exogenous expression in murine endothelial cells is sufficient to induce angioproliferative tumours similar to KS (Montaner *et al.*, 2003). The important role of the vGPCR in sarcomagenesis has led researchers to consider vGPCR expression and abortive/spontaneous lytic cycles (Chiou *et al.*, 2002; Ciufo *et al.*, 2001; Cannon *et al.*, 2005). How abortive lytic reactivation is induced and regulated is unclear, but has been documented *in vitro* (Nicholas *et al.*, 1998).

1.1.7. KSHV lytic DNA replication

Herpesvirus DNA replication is best characterised for HSV-1 whose DNA replication has been reconstituted *in vitro* (reviewed in Boehmer and Lehman, 1997; Lehman and Boehmer, 1999). Much of the machinery involved in DNA replication is conserved in all herpesviruses suggesting DNA replication proceeds by a conserved mechanism. KSHV ORFs 40/41, 6, 9, 44, 56 and 59 encoding a primase-helicase, single stranded DNA (ssDNA) binding protein, helicase, primase and processivity factor 8 (PF-8) respectively are capable of forming pseudo-replication compartments, even in the absence of viral DNA (Wu *et al.*, 2001). Herpesvirus DNA replication proceeds via a rolling circle mechanism generating concatermeric linear DNA.

Two origins of KSHV lytic replication have been defined (AuCoin *et al.*, 2002). K-bZIP and RTA are required for efficient replication initiation (AuCoin *et al.*, 2004;

Wang *et al.*, 2004c). The sequence of the lytic origin of DNA replication (ori-Lyt) is part of a functional KSHV promoter element, and RTA dependent transcription may be required for efficient replication initiation (Wang *et al.*, 2004c). Herpesviruses also encode a number of genes involved in DNA repair and nucleotide metabolism, such as uracil-DNA glycosidase and thymidine kinase. These enzymes are often necessary for replication *in vivo*, but are unnecessary *in vitro*. For example, murine gammaherpesvirus 68 with a disrupted thymidine kinase gene replicates normally *in vitro* but is severely attenuated *in vivo* (Coleman *et al.*, 2003).

1.1.8. KSHV assembly and morphogenesis

There is a paucity of published data regarding KSHV virion assembly and morphogenesis. Instead, the better-characterised events of HSV, HCMV and VZV assembly and morphogenesis will be considered here and are summarised in figure 1.1.5.

Herpesvirus capsid proteins synthesised in the cytoplasm are transported into the nucleus. Heterodimeric complexes of the scaffold protein and major capsid protein assemble into hexons and pentons (Wood *et al.*, 1997) generating a spherical procapsid which also contains minor capsid proteins (Newcomb *et al.*, 1999). A series of conformational changes induced by viral protease activity results in transformation of the procapsid into an icosahedral capsid structure (Trus *et al.*, 1996), a process that can be recapitulated *in vitro* (Newcomb *et al.*, 1994). DNA packaging involves the removal of scaffold proteins from the capsid and insertion of a single copy of linear genomic DNA. The details of this process are poorly defined but probably involve terminase proteins which recognise packaging signals in the viral DNA (Bogner *et al.*, 1998). Terminase proteins may also possess ATPase and endonuclease activity required for packaging and cleavage of concatameric genomes (Bronstein *et al.*, 1997; Dasgupta and Wilson, 1999).

The route of KSHV egress from the nucleus was, until recently, a controversial subject. Herpesviruses can be observed budding into different cellular membranes by electron microscopy (reviewed in Enquist *et al.*, 1998 and Roizman and Knipe, 2001). Using HSV-1 gD mutants with ER retention signals Minson and colleagues provided overwhelming evidence for an "Envelopment → Deenvelopment → Reenvelopment" pathway of herpesvirus egress (Skepper *et al.*, 2001).

Newly assembled nucleocapsids are able to bud through the inner nuclear membrane (Darlington and Moss, 1968). HSV-1 UL31 and UL34 are essential for this process (Chang *et al.*, 1997; Roller *et al.*, 2000) which is termed primary envelopment. UL34 is incorporated into the primary envelope whereas UL31 is involved in recruiting protein kinase C (PKC) which may phosphorylate the nuclear lamina easing primary envelopment (Muranyi *et al.*, 2002). Primary envelopment results in the generation of primary enveloped virions in the perinuclear space (Darlington and Moss, 1968). Consistent with their origin, primary enveloped virions have a different tegument structure and envelope consistency to mature virions (Granzow *et al.*, 1997).

Primary enveloped virions fuse with the outer membrane of the nuclear envelope releasing the nucleocapsid and primary tegument into the cytosol. The nucleocapsid then acquires a secondary envelope by budding into trans-Golgi vesicles (Siminoff and Menefee, 1968), a process termed secondary envelopment. Prior to secondary envelopment the nucleocapsid must acquire a mature tegument. The tegument is the structural component of the herpesvirion that varies the most between α, β, and γ-herpesviruses and few details of KSHV tegumentation are known. The details of tegument assembly will not be considered further here, see Mettenleiter, 2004 for an extensive review.

The nucleocapsid surrounded by a mature tegument undergoes secondary envelopment generating a mature herpesvirus virion inside a trans-Golgi vesicle. Viral glycoproteins are enriched at the site of secondary envelopment through the action of gM (Crump *et al.*, 2004). This generates a mature viral envelope rich in viral glycoproteins. Vesicle fusion with the plasma membrane liberates the mature virion, which can potentially infect new target cells and be transmitted to new hosts.

1.1.9. KSHV Transmission

Current understanding of the KSHV life cycle is at best fragmented. Elucidation of the lifecycle has been hampered by the lack of a KSHV animal model, poor *in vitro* infectivity, mild symptomatic infection of the immunocompetent host and large variations in the serological and molecular techniques used to monitor infections *in vivo*. In the absence of an accepted coherent model of the KSHV lifecycle, data

concerning the transmission and tropism of KSHV *in vivo* are discussed here and in section 1.1.10.

Many epidemiological studies of KSHV have focussed on KSHV transmission (reviewed in Corey *et al.*, 2002; Henke-Gendo and Schulz 2004). These studies have focussed on maternal, sexual, blood borne and transplant associated transmission and these are considered in turn. Maternal transmission of KSHV is supported by several epidemiological studies (Plancoulaine *et al.*, 2000; Sitas *et al.*, 1999). In regions where KSHV is endemic, the probability of being seropositive increases with age and maternal antibody titre for KSHV (Ziegler *et al.*, 2003; Sitas *et al.*, 1999).

Sexual transmission of KSHV continues to be an extremely controversial issue. It is clear that among homosexual men in Europe and North America promiscuity, HIV-infection and a history of other sexually transmitted diseases are behavioural risk factors associated with KSHV seropositivity (Reviewed in Schulz, 2000). However, the risk factors associated with heterosexual activities are far less clear. Several studies suggest heterosexual sexual transmission is unlikely, finding no increased transmission between spouses (Plancoulaine *et al.*, 2000), no association with other sexually transmitted diseases (Fernandez *et al.*, 2002) and no increased transmission to sex workers (Marcelin *et al.*, 2002). However, studies that directly contradict these observations have also been published (Mbulaiteye *et al.*, 2003; Baeten *et al.*, 2002; Eltom *et al.*, 2002).

KSHV DNA is readily detectable in serum from blood donors (Enbom *et al.*, 2002) however there have been no well-documented cases of transfusion-borne KSHV infection (Operskalski *et al.*, 1997), although possible cases have been highlighted (Dollard *et al.*, 2005).

Many years before the discovery of KSHV, an association between KS and transplant recipients was apparent (Harwood *et al.*, 1979). This association is mainly due to reactivation of KSHV, which can lead to KS, in immunosuppressed patients (Cattani *et al.*, 2001). Transmission of KSHV from donor to recipient patients has also been documented (Parravicini *et al.*, 1997a). More recently, transmission of KSHV positive donor-derived progenitor cells was demonstrated to initiate sarcomagenesis in transplant recipients. These transplanted cells remained in KS lesions of recipient

patients (Barozzi *et al.*, 2003), suggesting that transmission of other tumours to immunocompromised individuals may be possible (Moore, 2003).

Although post transplant KS is important in the clinic, data concerning KSHV transmission suggests that saliva is the most common source of transmissible KSHV. KSHV DNA is detected at a far higher frequency in oropharyngeal samples (30%) than in anal or genital samples (1%) of KSHV-positive men who had sex with men (Pauk *et al.*, 2000). As many as $1x10^6$ copies per ml of KSHV DNA have been detected in saliva (Koelle *et al.*, 1997) and saliva is the only mucosal secretion in which KSHV virions have been identified (Vieira *et al.*, 1997). Salivary transmission has also been suggested to explain epidemiological data supporting transmission within homosexual male risk groups (Pauk *et al.*, 2000). However, it is difficult to reconcile this explanation with the lack of association between KSHV transmission and heterosexual risk groups. It is interesting to note that the prevalence of KSHV is far lower than other herpesviruses whose transmission is predominantly through salivary contact. EBV, CMV, HHV-6 and HHV-7 are nearly ubiquitous in human populations whereas KSHV seroprevalence ranges from 1-10% in most of Europe and Asia, rises to approximately 25% in the Mediterranean and the Middle East, and approaches 70% in regions of sub-Saharan Africa (reviewed in Iscovich *et al.*, 2000). The factors accounting for the lower seroprevalence of KSHV relative to other herpesviruses and the wide geographic variation in prevalence are unclear.

1.1.10. KSHV cellular tropism *in vivo*

Following transmission of KSHV, the initial cell-target of infection is not clear although oral epithelial cells have been suggested as a possible permissive cell-type (Duus *et al.*, 2004). Initial infection seems to be accompanied by viraemia both in immunocompetent children (Kasolo *et al.*, 1997) and immunocompromised adults (Wang *et al.*, 2001a; Goudsmit *et al.*, 2000). This initial viraemia is associated with a febrile illness and mild lymphadenopathy (Wang *et al.*, 2001a; Kasolo *et al.*, 1997). Although the default outcome of KSHV infection *in vitro* with PEL-derived and recombinant KSHV seems to be latent infection (Bechtel *et al.*, 2003), virions obtained from saliva may favour entry into a lytic cycle (Gasperini *et al.*, 2005; Duus *et al.*, 2004), possibly accounting for this initial burst of viraemia.

Following initial viraemia, latent infection of B-cells can be detected (Harrington *et al.*, 1996; Mesri *et al.*, 1996; Campbell *et al.*, 2005). The frequency of infected cells is too low for the exact B-cell subset supporting latency to be identified. However, B-cells are the likely latent reservoir of KSHV *in vivo* and are the only cell-type that latent KSHV has been detected in healthy individuals (Campbell *et al.*, 2005).

KSHV infection can also be detected in malignancies associated with KSHV, discussed in sections 1.1.16-1.1.18. Infected B-cells can be detected in PEL (Cesarman *et al.*, 1995a) and multicentric Castleman's disease (MCD) (Soulier *et al.*, 1995), whereas infected endothelial cells (Boshoff *et al.*, 1995) and to a lesser extent monocytes can be detected in KS (Blasig *et al.*, 1997). It is important to note that KSHV infection of endothelial cells has not been reported in immunocompetent, healthy individuals and is probably not a requirement of the KSHV lifecycle.

1.1.11. Cells supporting KSHV infection *in vitro*

In accordance with the widespread expression of characterised KSHV receptors (reviewed in Page *et al.*, 1997; Kreidberg *et al.*, 2000) the range of cells supporting KSHV infection *in vitro* is wider than the apparent tropism *in vivo* (table 1.1.11). All the known targets of KSHV infection *in vivo* support KSHV infection *in vitro* as well as a range of adherent mammalian cell-lines.

The most intriguing facet of KSHV infection *in vitro* is the difficulty in infecting cultured lymphoma-derived cell-lines. The EBV negative Burkitt's lymphoma derived cell-line BJAB (Menezes *et al.*, 1975) is the only B-cell line in which KSHV infection has been reported *in vitro* (Naranatt *et al.*, 2004; Gasperini et al., 2005). Only two groups have achieved BJAB infection *in vitro* and several groups have been unable to achieve BJAB infection (Chen and Lagunoff, 2005; Bechtel *et al.*, 2003; Blackbourn *et al.*, 2000b; Renne *et al.*, 1998). There are numerous explanations that could explain this apparent discrepancy, most of which concern variation in the source of KSHV, method of infection and assays used to monitor infection. Whatever the explanation, it is clear that B-cell lines appear to be largely recalcitrant to KSHV infection *in vitro* a fact apparently at odds with the cellular tropism of KSHV *in vivo*.

Table 1.1.11. The host range of KSHV in cultured cells

Cell type/line	Abbreviation	References
Primary CD19+ B-cells		Mesri *et al.*, 1996
Human Peripheral blood mononuclear cells	Human PBMC	Blackbourn *et al.*, 2000b
Baboon Peripheral blood mononuclear cells	Human PBMC	Blackbourn *et al.*, 2000b
Rhesus macaque Peripheral blood mononuclear cells	Human PBMC	Blackbourn *et al.*, 2000b
Human monocyte derived macrophages		Blackbourn *et al.*, 2000b
Human umbilical cord mononuclear cells	UCMC	Blackbourn *et al.*, 2000b
EBV transformed lymphoblastoid cell-lines	LCL	Blackbourn *et al.*, 2000b
BJAB-derived B-cell line	BFF	Gasperini et al., 2005
Dermal microvascular endothelial cells	DMVEC	Flore *et al.*, 1998
Immortalised dermal microvascular endothelial cells	t-DMVEC	Blackbourn *et al.*, 2000b
Transformed dermal microvascular endothelial cells	DMVEC	Moses *et al.*, 1999
Mesoendothelial cells	RMEC-1	Blackbourn *et al.*, 2000b
Brain-derived endothelial cell-line	BB19	Blackbourn *et al.*, 2000b
Transformed human brain endothelial cells	181GB1-4	Renne *et al.*, 1998
Neonatal capillary endothelial cells		Renne *et al.*, 1998
Chinese hamster ovary cell-line	CHO	Bechtel *et al.*, 2003
Owl monkey kidney cells	OMK637	Renne *et al.*, 1998
Human epithelial kidney cells	293	Renne *et al.*, 1998
Baby hamster kidney cells	BHK-21	Renne *et al.*, 1998
Prostate cancer derived cell line	Ln-Cap	Renne *et al.*, 1998
Human lung carcinoma cells	A549	Renne *et al.*, 1998
Chediak-Higachi syndrome cells	CHELI	Renne *et al.*, 1998
Squamous cell carcinoma of the tongue	SCC15	Renne *et al.*, 1998
Human foreskin fibroblasts	HFF	Bechtel *et al.*, 2003
KS-derived endothelial cell-line (KSHV –ve)	SLK	Bechtel *et al.*, 2003
African green monkey kidney cell-line	CV-1	Bechtel *et al.*, 2003
Human cervical carcinoma cell-line	HeLa	Bechtel *et al.*, 2003
Murine fibroblast cell-line	3T3	Bechtel *et al.*, 2003
Quail fibroblast cell-line	QT-6	Bechtel *et al.*, 2003

Adapted from; Renne *et al.*, 1998; Blackbourn *et al.*, 2000b; Corey *et al.*, 2001; Bechtel *et al.*, 2003.

1.1.12. Establishing latency

Latency is a fascinating aspect of herpesvirology. The concept of latency was first applied to herpesviruses in the late 1930s. Burnet and Williams stated that, "Herpes simplex infections, however, once contracted, seem to persist for life. The virus remains for the most part latent; but under the stimulus of trauma, fever, and so forth it may at any time be called into activity and provoke a visible herpetic lesion" (Burnet and Williams, 1939). The observation that herpesviruses can establish lifelong latent infections has stood the test of time and all known human herpesviruses can establish such infections. There are many different operational and clinical definitions of herpesvirus latency, but all definitions concur that latency is an initially nonproductive herpesvirus infection that retains the capacity to reactivate and produce infectious virions (Roizman and Knipe, 2001; Kieff and Rickinson, 2001; Mocarski and Courcelle, 2001).

The study of herpesviral latency began with human alphaherpesviruses. Reactivation of alphaherpesviruses from latency frequently, but not necessarily, results in the generation of herpetic lesions (reviewed in Efstathiou and Preston, 2005; Kennedy, 2002). This has lead to the term 'reactivation' being used to describe both clinical recurrence of symptoms and the molecular switch from latent to lytic gene expression programmes. For clarity, this thesis uses the term 'reactivation' to describe, solely, the molecular switch from a latent to a lytic gene expression programme.

Following cellular infection herpesviruses either enter a lytic or latent gene expression programme. How the virus is directed into either programme is a subject of great interest. For alphaherpesvirus, the nature of the host cell seems critical for governing which programme the virus enters. For example HSV-1 enters a lytic cycle upon infection of oral mucosal epithelium but upon infection of sensory neurons innervating the site of infection, HSV-1 enters a latent gene expression programme (Bastian *et al.*, 1972). It is widely accepted that HSV-1 latency occurs by default if the lytic cycle is not initiated following infection (Reviewed in Roizman and Knipe, 2001). This model is strongly supported by the observation that impairing alpha gene expression following infection can switch HSV-1 from a lytic to a latent gene expression programme *in vitro* (Samaniego *et al.*, 1998; Marshall *et al.*, 2000). Exactly how alpha gene expression is prevented *in vivo* following infection of neurons is unclear, but probably involves the

host proteins Octamer binding protein (Oct-1) and host cell factor (HCF). Oct-1 and HCF bind to VP16 (Stern *et al.*, 1989; Gerster and Roeder, 1988) forming a heterotrimeric complex capable of promoting immediate early gene expression (reviewed in Wysocka and Herr, 2003). It has been suggested that the cytoplasmic location of HCF in neurons prevents active complex formation in the nucleus, resulting in greatly reduced alpha gene expression and initiation of a latent gene expression programme (Kristie *et al.*, 1999; La Boissiere *et al.*, 1999).

The establishment of latency for human betaherpesviruses is not as well characterised as human alphaherpesviruses. It is clear that HCMV can establish a latent infection in lineage committed myeloid cells including progenitors of granulocytes, macrophages and dendritic cells (Hahn *et al.*, 1998). The mechanism determining latent infection of these cells is poorly characterised, but similarly to alphaherpesviruses a lack of alpha gene expression is implicated in this process. Transcriptional repressors expressed in myeloid progenitors are capable of reducing gene expression from the HCMV major immediate early promoter (reviewed in Meier and Stinski, 1996), possibly resulting in non-productive HCMV latency by default (reviewed in Sinclair, 2000).

Herpesvirus latency is best characterised for human gammaherpesviruses and EBV latency is discussed in sections 1.2.7. In contrast to alpha and beta herpesviruses, minimal KSHV alpha gene expression is not the decisive factor in determining entry of KSHV into a latent gene expression programme. In a fascinating study, Chandran and colleagues revealed that entry into an abortive lytic gene expression programme precedes the latency programme (Krishnan *et al.*, 2004). KSHV receptor binding (Sharma-Walia *et al.*, 2005; Lu *et al.*, 2005b) and constituents of the virion (Bechtel *et al.*, 2005a; 2005b) seem to result in the restricted expression of lytic genes following infection *in vitro* (Krishnan *et al.*, 2004). This gene expression is transient and latency ensues. The mechanism through which latency interrupts the lytic cycle is uncertain, but induction of latency-associated nuclear antigen (LANA) expression by RTA (Lan *et al.*, 2005b; Matsumura *et al.*, 2005) and subsequent repression of RTA expression by LANA (Lan *et al.*, 2004) has been suggested as a potential model (Lan *et al.*, 2005b).

1.1.13. KSHV latent gene expression

A key feature of latency is the restricted expression of viral genes, which minimises the number of viral epitopes presented to the immune system assisting in immune evasion

(reviewed in Stevenson, 2004). Latent infection by KSHV is predominant in KS, PEL and MCD and the contribution of KSHV genes, expressed during latency, to tumourigenesis has been the focus of much research (reviewed in Cathomas, 2003).

In all documented latent infections KSHV expresses LANA (Kedes *et al.*, 1997), viral Fas-associated death domain (FADD) interleukin-1β-converting enzyme (FLICE) inhibitory protein (vFLIP) (Grundhoff and Ganem, 2001; Low *et al.*, 2001) and viral-cyclin (v-cyclin) (Cesarman *et al.*, 1996). These proteins, expressed from the major latency locus (Dittmer *et al.*, 1998), are encoded by differentially spliced polycistronic mRNAs usually originating from a common promoter (Talbot *et al.*, 1999; Pearce *et al.*, 2005). This constitutively active promoter is particularly strong in B-cells (Jeong *et al.*, 2002), upregulated during S-phase (Sarid *et al.*, 1999) and autoactivated by LANA (Renne *et al.*, 2001).

Table 1.1.13.1. KSHV genes expressed during latency.

Gene	Protein	Function	References
ORF71	vFLIP	Inhibits FAS-mediated apoptosis	Djerbi *et al.*, 1999
		Activates NFκB	Liu *et al.*, 2002
ORF72	v-cyclin	Constitutively activates CDK6	Swanton *et al.*, 1997
ORF73	LANA	Episome maintenance	Ballestas et al., 1999
		Binds p53 and represses transcription	Friborg *et al.*, 1999
		Transcriptional regulation	Radkov *et al.*, 2000
ORF K2	vIL-6	Autocrine growth factor	Moore *et al.*, 1996a
		Induces cellular IL-6 expression	An *et al.*, 2002
ORF K10.5	LANA2	Binds p53 and represses transcription	Rivas *et al.*, 2001
		Inhibits PKR	Esteban *et al.*, 2003
ORF K11.1	vIRF2	Inhibits PKR	Burysek and Pitha, 2001
		Inhibits IRF-1 and IRF-7 mediated transcription	Burysek *et al.*, 1999
ORF K12	Kaposin	Stabilises cytokine transcripts	McCormick and Ganem, 2005
		Source of miRNAs	Cai *et al.*, 2005
ORF K15	K15	Activates Mitogen activated protein kinases and nuclear factor kappa B	Brinkmann *et al.*, 2003

In addition to genes transcribed from the major latency locus, several other KSHV genes are commonly transcribed during latency. These genes and their functions are listed in table 1.1.13.1. It is important when considering latent gene expression to remember that genes expressed during latency are not exclusive to latency. Indeed,

viral interleukin 6 (vIL-6) (Katano *et al.*, 2000), K12 (Sadler *et al.*, 1999), LANA, vFLIP and v-cyclin (Jenner *et al.*, 2001; Matsumura *et al.*, 2005) all increase their expression following lytic induction *in vitro*.

KSHV latent infections are not uniform and gene expression profiles vary in different situations. By analogy to EBV it is possible that KSHV has tightly regulated latent gene expression programmes suited to its different cellular lifestyles. Characterisation of KSHV latent gene expression has been hampered by the inability of KSHV to form lymphoblastoid cell-lines *in vitro* (Kliche *et al.*, 1998) and the rarity of KSHV infected B-cells in healthy individuals. However, KSHV gene expression can be monitored in KS biopsies, MCD biopsies, PEL cells and PEL cell-lines. This is summarised in table 1.1.13.2.

Table 1.1.13.2. Characterised latent gene expression.

Gene/ protein	Expressed during latency in KS?	Expressed during latency in PEL?	Expressed in KSHV associated MCD?
ORF71/ vFLIP	Yes (Sturzl *et al.*, 1999)	Yes (Low *et al.*, 2000)	
ORF72/ v-cyclin	Yes (Reed *et al.*, 1998)	Yes (Platt *et al.*, 2000)	
ORF73/ LANA	Yes (Rainbow *et al.*, 1997)	Yes (Rainbow *et al.*, 1997)	Yes (Dupin *et al.*, 1999)
ORF K2/ vIL-6		Yes (Staskus *et al.*, 1999)	Yes (Staskus *et al.*, 1999)
ORF K10.5/ LANA2		Yes (Rivas *et al.*, 2001)	Yes (Rivas *et al.*, 2001)
ORF K11.1/ vIRF2		Yes (Burysek and Pitha, 2001)	
ORF K12/ Kaposin	Yes (Zhong *et al.*, 1996)	Yes (Muralidhar *et al.*, 1998)	
ORF K15/ K15		Yes (Glenn *et al.*, 1999)	Yes (Sharp *et al.*, 2002)

The characterisation of KSHV genes as latent can be problematic. A small proportion of cells in PEL, PEL cell-lines and KS biopsies express lytic genes and are in a lytic or

abortive lytic gene expression programme (Parravicini *et al.*, 2000). Although only a minority of cells express these genes, they can be expressed at such a level that they dominate lysates from a population of cells. An example of this is the polyadenylated nuclear transcript (PAN) also described as nuclear transcript 1 (nut-1) or transcript 1.1 (T1.1). PAN was initially described as a latent gene (Zhong *et al.*, 1996), but was subsequently recognised as being expressed at high levels in cells supporting lytic replication (Staskus *et al.*, 1997; Zhong *et al.*, 1997; Sun *et al.*, 1999). To qualify as being expressed during latency, a larger proportion of cells than those expressing lytic genes must be identified. This has led to *in situ* hybridisation (Reed *et al.*, 1998) and immunohistochemistry (Dupin *et al.*, 1999) being adopted as the techniques of choice for identifying latent gene expression.

Perhaps the most interesting observation from KSHV-associated neoplasms is the differential regulation of LANA2 expression. LANA2 is expressed during latency in B-cells, but is exclusively a lytic gene in KS biopsies (Rivas *et al.*, 2001). Although the mechanism and benefit of this differential expression are unclear, it is proof of principle that KSHV can adopt multiple latency programmes. Different latency programmes could explain some controversial aspects of latent gene expression. For example vIL-6, whose expression is important in the pathogenesis of KSHV-associated MCD (Parravicini *et al.*, 1997b), has been classified as both a lytic gene (Katano *et al.*, 2000) and a latent gene (Cannon *et al.*, 2000). Although KSHV infected cells in MCD can express vIL-6 (Parravicini et al., 1997b; 2000), it is uncertain whether this is latent or lytic cycle expression. The inclusion of vIL-6 in table 1.1.13.2 is therefore controversial, but it is clear that vIL-6 can be expressed during latency in PEL cell-lines (Cannon *et al.*, 2000) and that expression can be modulated in the absence of lytic replication (Chatterjee *et al.*, 2002; Deng *et al.*, 2003; Chang *et al.*, 2005a).

There is great potential for the individual regulation of KSHV gene expression during latency. KSHV ORF34, a gene of unknown function, is one such gene. Hypoxia response elements in the ORF34 promoter facilitate the upregulation of ORF34 in a hypoxia inducible factor-1 alpha (HIF-1 alpha) and HIF-2 alpha dependent fashion (Haque *et al.*, 2003). It is possible that other KSHV genes are independently controlled and expressed during latency *in vivo*. Indeed, notch signalling has recently been demonstrated to induce the expression of multiple viral genes without initiating a lytic replication programme (Chang *et al.*, 2005a). In light of the apparent flexibility of

KSHV gene expression, it is tempting to speculate that KSHV might adopt a variety of latent gene expression programmes during its life cycle. A strategy analogous to that which is used by EBV (reviewed in Kieff and Rickinson, 2001; Thorley-Lawson, 2001).

1.1.14. KSHV reactivation from latency

Reactivation from latency in B-cells is an important facet of the KSHV lifecycle. A better understanding of KSHV reactivation could aid our understanding of disease progression and viral transmission. The importance of lytic gene expression in KS and MCD (Discussed in section 1.2.7) suggests that the study of reactivation could identify new targets of therapeutic interest for the treatment of KS and KSHV-associated MCD.

Waves of virus shedding can be detected in the saliva of KSHV positive individuals (Pauk *et al.*, 2000) and reactivation of KSHV from latency in B-cells is probably responsible for initiating waves of transient viraemia, a process important in viral transmission.

As well as transmission, reactivation from latency is important in KSHV pathogenesis. KSHV infection may precede KS development by decades (Cattani *et al.*, 2001) and reactivation from latency in B-cells seems critical for progression from asymptomatic infection to KS. Treatment of existing KS lesions with ganciclovir, an anti-herpetic drug that inhibits lytic replication (Cheng *et al.*, 1983; Cannon *et al.*, 1999), has little effect on disease progression (Robles *et al.*, 1999). This is probably because latent and early gene expression drives tumourigenesis (reviewed in Hayward, 2003), both of which are insensitive to ganciclovir treatment (Lu *et al.*, 2004). Although unable to facilitate resolution of KS lesions, ganciclovir is remarkably effective at reducing the risk of developing KS. Treatment of HIV-positive individuals, suffering from cytomegalovirus retinitis, with oral or intravenous ganciclovir reduced the risk of developing KS by 75% or 93% respectively (Martin *et al.*, 1999), presumably through preventing reactivation of latent KSHV. These data are consistent with the observation that an increased KSHV viral load in PBMC is associated with progression to KS in previously asymptomatic carriers (Ambroziak *et al.*, 1995).

Lytic reactivation may also be important in the pathogenesis of KSHV-associated MCD (Parravicini *et al.*, 2000). Consistent with this hypothesis, treatment of MCD patients

with ganciclovir can reduce the frequency of episodic flares (Casper *et al.*, 2004) highlighting the importance of reactivation in this disease.

Although no direct role for KSHV lytic replication in the development or pathogenesis of PEL has been described, understanding reactivation could be important in the treatment of PEL. Inducing lytic replication in PEL cells could make anti-herpetic drugs, such as ganciclovir, useful therapeutic agents in treating this neoplasm. For example, valproate is a histone deacetylase inhibitor licensed for use in the treatment of various neurological disorders. Valproate can reactivate latent KSHV at concentrations similar to those observed in the clinic (Shaw *et al.*, 2000). A combination of Valproate and ganciclovir has proved effective in blocking KSHV lytic replication and inducing apoptosis of PEL cells *in vitro* (Klass *et al.*, 2005). The clinical utility of this strategy has already been observed in a patient suffering from post-transplant lymphoproliferative disease, a B-cell neoplasm associated with EBV. EBV lytic replication was induced through administration of arginine-butyrate and blocked through administration of ganciclovir. This resulted in substantial tumour necrosis (Mentzer *et al.*, 1998).

Over 20 situations resulting in the reactivation of KSHV from latency *in vitro* have been described and these are summarised in table 1.1.14.1. All of these stimuli have been shown to, or are presumed to, act through inducing RTA expression (reviewed in West and Wood, 2003). It is widely accepted that expression of RTA is both necessary (Xu *et al.*, 2005) and sufficient (Lukac *et al.*, 1998) to initiate KSHV lytic replication in PEL cell-lines. Once expressed, RTA is able to autoactivate the ORF50 promoter (Deng *et al.*, 2000) resulting in substantial amplification of any stimulus that induces RTA expression.

The proportion of cells harbouring KSHV that reactivate following stimulation varies depending on which stimulus is used (Chang *et al.*, 2000). This could be due to cellular factors or viral factors. For example, 10-fold more PEL cells synchronised in S-phase reactivate following TPA treatment relative to asynchronous controls (McAllister *et al.*, 2005) and cells harbouring the naturally occurring KSHV deletion mutant KV-1 are unable reactivate *in vitro* (Deng *et al.*, 2004) As well as variation within cell populations, different cell types respond differently to each stimulus. For example TPA causes maximal induction in most PEL cell-lines, but HBL-6 and HH-B2 PEL cell-lines

Table 1.1.14.1. Stimuli reported to reactivate latent KSHV.

Stimulus	Mechanism	Reference
12-O-tetradecanoyl phorbol-13-acetate (TPA)	Requires activation of protein kinase Cδ (Deutsch *et al.*, 2004) and involves activating protein 1 (Wang *et al.*, 2004b)	Renne *et al.*, 1996b Moore *et al.*, 1996b
Sodium butyrate	Inhibits histone deacetylases causing chromatin remodelling of ORF50 promoter (Lu *et al.*, 2003) and recruitment of stimulating protein 1 (Sp1) and Sp3 complexes (Ye *et al.*, 2005)	Miller *et al.*, 1996 Miller *et al.*, 1997
Ionomycin	Mobilises Ca^{2+} and reactivation is dependent on calcineurin (Zoeteweij *et al.*, 2001)	Chang *et al.*, 2000
Thapsigargin	Mobilises Ca^{2+} and reactivation is dependent on calcineurin (Zoeteweij *et al.*, 2001)	Zoeteweij *et al.*, 2001
5-azacytidine	Demethylation of ORF50 promoter (Chen *et al.*, 2001)	Chen *et al.*, 2001
Prostratin		Brown *et al.*, 2005
Bortezomib		Brown *et al.*, 2005
Valproic acid	Inhibits histone deacetylases (reviewed in Blaheta *et al.*, 2005)	Shaw *et al.*, 2000
Hypoxia	Hypoxia inducible factors (HIFs) activate the ORF50 promoter (Haque *et al.*, 2003)	Davis *et al.*, 2001
Epithelial differentiation		Johnson *et al.*, 2005
Adrenalin and Noradrenalin	Effect dependent upon protein kinase A	Chang *et al.*, 2005b
Interferon-γ		Blackbourn *et al.*, 2000a Chang *et al.*, 2000 Mercader *et al.*, 2000 Monini *et al.*, 1999
Oncostatin M		Mercader *et al.*, 2000
Hepatocyte growth factor/ scatter factor		Mercader *et al.*, 2000
Interleukin-6		Song *et al.*, 2002
Trichostatin A	Inhibits histone deacetylases	Lu *et al.*, 2003
Hydrocortisone		Hudnall *et al.*, 1999
HIV-1 tat		Harrington *et al.*, 1997
HVS RTA		Goodwin et al., 2001
KSHV RTA	Auto activates RTA expression (Deng *et al.*, 2000)	Lukac *et al.*, 1998
MHV68 RTA		Damania *et al.*, 2004
RRV RTA		Damania *et al.*, 2004
cFOS-cJUN heterodimer	Transactivate ORF50 promoter (Wang *et al.*, 2004b)	Wang *et al.*, 2004b
HHV6 coinfection		Lu *et al.*, 2005a
HIV-1 coinfection	Possibly HIV-1 tat protein	Merat *et al.*, 2002
HCMV coinfection		Vieira *et al.*, 2001

seem refractory to induction with TPA (Chiou *et al.*, 2002; Ye *et al.*, 2005). With the exception of interferon-γ, the proportion of cells reactivating in response to cytokines and hormones is typically low. Indeed, the ability of hydrocortisone and interleukin-6 to induce reactivation of KSHV is not consistently reported (Zoeteweij *et al.*, 2002; Chang *et al.*, 2000) even using the same cell-line (Song *et al.*, 2002; Chang *et al.*, 2000). Both oncostatin M and IL-6 are produced by PEL cell-lines (Drexler *et al.*, 1999) and could be involved in initiating the low level 'spontaneous reactivation' observed in these cells under standard culture conditions.

The reactivation mechanisms described to date demonstrate that many different pathways including; activation of protein kinase Cδ (PKCδ) (Deutsch *et al.*, 2004), mobilisation of intracellular calcium (Zoeteweij *et al.*, 2001), histone acetylation (Lu *et al.*, 2003), expression of hypoxia inducible factors (Haque *et al.*, 2003) and demethylation of the ORF50 promoter (Chen *et al.*, 2001) can induce ORF50 expression *in vitro*. The existence of multiple pathways effecting reactivation *in vitro* suggests that latent KSHV retains the capacity to reactivate in a multitude of different scenarios *in vivo*. However, despite so many reactivation stimuli being described, it is unclear which pathways are physiologically relevant and which stimuli activate many pathways *in vivo*. The most convincing study analysing reactivation *in vivo* observed that DNA methylation patterns of the ORF50 promoter vary between asymptomatic latent-infections and biopsies from KSHV-associated disease *in vivo* (Chen *et al.*, 2001). However, the mechanism(s) resulting in promoter demethylation *in vivo* and a causal connection to reactivation *in vivo* remain elusive.

1.1.15. RTA and the ORF50 promoter

RTA has a pivotal role in the lifecycle of KSHV. Once expressed, RTA amplifies its own expression (Deng *et al.*, 2000) and participates in complexes activating multiple viral and cellular genes (reviewed in West and Wood, 2003), initiating a tightly regulated gene expression cascade eventually resulting in the production of progeny virions. RTA can therefore be considered as a 'molecular switch' whose expression must be suppressed during latency and activated to initiate lytic replication. The decision between latent and lytic gene expression programmes can therefore be considered in terms of the transcriptional activation state of the ORF50 promoter.

Sequence specific transcription factors act as a bridge between regulatory DNA sequences and the core transcriptional machinery and chromatin remodelling factors (reviewed in Kadonaga, 2004). Because of their decisive role in reactivation, transcription factor complexes involving RTA and the ORF50 promoter are an important area of KSHV research. Much of the work in this field involves the study of complexes associated with RTA. Whilst these complexes are essential for amplifying and maintaining initial activation of the ORF50 promoter, they are not informative about the complex(es) involved in initiating reactivation in the absence of RTA. In addition, RTA is involved in the transcriptional regulation of multiple viral and cellular promoters and interacts with a multitude of viral and cellular factors. This makes it difficult to generalise about transcription factor complexes involving RTA. Indeed, different complexes involving RTA facilitate the differential regulation of multiple RTA responsive promoters, an essential feature of the KSHV lytic gene expression cascade. The reported interactions directly implicated in the control of ORF50 expression are summarised in table 1.1.15.1.

Seven transcription factors, in addition to RTA, have been described which initiate ORF50 expression when exogenously expressed *in vitro* (Wang *et al.*, 2003; Haque *et al.*, 2003; Wang *et al.*, 2004b; Malik *et al.*, 2004) and are listed in table 1.1.15.1. KSHV ORF57 is not expressed during latency (Lukac *et al.*, 1999) and is unlikely to play a part in initiating ORF50 expression. Of the remaining transcription factors, only HIF-2 has a defined mechanism resulting in its expression in B-cells *in vivo*. B-cells are frequently exposed to hypoxic environments because lymphoid organs have a much lower oxygen tension than blood (Kojima *et al.*, 2003). The physiological events capable of modulating CCAAT/ Enhancer-binding protein-α (C/EBPα), activating protein 1 (AP-1), cAMP response element binding protein (CBP) and specificity protein 1 (SP-1) activity *in vivo* and their possible role in KSHV reactivation remain unclear.

Following chemical induction of lytic replication *in vitro* many factors involved with transcriptional regulation of the ORF50 promoter have been identified. Most of these factors interact with RTA although Oct-1 is a notable exception (Sakakibara *et al*, 2001). Although a potent activator of ORF50 expression, RTA is unable to bind the ORF50 promoter directly (Sakakibara *et al*, 2001). Protein-protein interactions

therefore seem essential to direct RTA to the ORF50 promoter. A particularly interesting interaction directing RTA to the ORF50 promoter is with recombination

Table 1.1.15.1. Transcription factors reportedly involved in activating transcription from the ORF50 promoter.

Protein	Capable of transactivating the ORF50 promoter in the absence of RTA?	Capable of cooperating with RTA to activate the ORF50 promoter?
cFOS-cJUN/ activating protein 1 (AP-1)	Exogenously expressed AP-1 can induce RTA expression in BCBL-1 cells (Wang *et al.*, 2004b)	Interacts with RTA enhancing RTA-mediated transactivation (Wang *et al.*, 2004b)
cJun	Can transactivate the ORF50 promoter in cotransfection experiments with reporter gene constructs (Gwack *et al.*, 2001)	
CCAAT/ Enhancer-binding protein-α (C/EBPα)	Exogenously expressed C/EBPα can induce RTA expression in BCBL-1 cells (Wang *et al.*, 2003)	Cooperates with RTA in activating the ORF50 promoter (Wang *et al.*, 2003)
ORF57	Can transactivate the ORF50 promoter in cotransfection experiments with reporter gene constructs (Malik *et al.*, 2004)	Interacts with RTA enhancing RTA mediated transactivation (Malik *et al.*, 2004)
Hypoxia inducible factor-2 (HIF-2)	Can transactivate the ORF50 promoter in cotransfection experiments with reporter gene constructs (Haque *et al.*, 2003).	
Specificity protein 1 (SP-1)	Can transactivate the ORF50 promoter in cotransfection experiments with reporter gene constructs (Ye *et al.*, 2005).	
Recombination signal-binding protein 1 for J-kappa (RBP-Jκ)		Interacts with RTA helping to target RTA transactivation Liang *et al.*, 2002)
MGC2663/ KSHV-RTA binding protein (K-RBP)		Interacts with RTA weakly enhancing RTA-mediated transactivation (Wang *et al.*, 2001b)
Octamer-binding protein 1 (Oct-1)		Oct-1 binds octamer sequences in the ORF50 promoter and enhances RTA-mediated transactivation (Sakakibara *et al.*, 2001)
cAMP response element (CRE)-binding protein (CBP)	Can transactivate the ORF50 promoter in cotransfection experiments with reporter gene constructs (Lu *et al.*, 2003)	CBP binds RTA and enhances RTA mediated transcription (Gwack *et al.*, 2001)

signal-binding protein 1 for J-kappa (RBP-Jκ) also known as C-promoter binding factor (CBF). RBP-Jκ is a transcriptional repressor which is the major target of Notch signalling (reviewed in Mumm and Kopan, 2000). Notch signalling results in the displacement of corepressor complexes from RBP-Jκ and the subsequent recruitment of coactivator complexes. Similarly, when RTA is recruited to RBP-Jκ it results in the formation of a transcriptional activating complex in a Notch ligand-independent fashion (Liang *et al.*, 2002). RBP-Jk may therefore help maintain latency by repressing ORF50 transcription whilst remaining essential for reactivation (Liang *et al.*, 2003), by directing RTA to the ORF50 promoter and other viral promoters following RTA induction (Liang *et al.*, 2002; Chang *et al.*, 2005c).

An important process in transcriptional regulation is chromatin remodelling. Analysis of the ORF50 promoter in PEL cell-lines harbouring latent KSHV indicates that the ORF50 promoter is in a region of densely packed chromatin associated with histone deacetylases (Lu *et al.*, 2003). Following chemical induction of the lytic cycle with sodium butyrate or trichostatin A the chromatin conformation is transformed to a more relaxed acetylated state (Lu *et al.*, 2003). RTA is able to interact with cAMP response element (CRE)-binding protein (CBP) (Gwack *et al.*, 2001), a protein with intrinsic histone acetyl transferase (HAT) activity (Bannister and Kouzarides, 1996), potentially recruiting HAT activity to the ORF50 promoter. Following induction of KSHV lytic replication *in vitro*, both CBP and p300, another protein with intrinsic HAT activity (Ogryzko *et al.*, 1996), can be detected in complexes on the ORF50 promoter (Ye *et al.*, 2005).

The importance of ORF50 promoter de-repression is further supported by the DNA methylation state of the ORF50 promoter *in vivo*. The ORF50 promoter in asymptomatic KSHV-positive individuals is heavily methylated (Chen *et al.*, 2001). Methyl CPG binding proteins (MeCPs) bind methylated DNA and are capable of recruiting HDACs (Nan *et al.*, 1998), helping to maintain a transcriptionally repressed state. Treating PEL cell-lines with 5-azacytidine, a demethylating agent, leads to reactivation of KSHV *in vitro* (Chen *et al.*, 2001), suggesting that demethylation plays an important role in activating ORF50 expression *in vivo*. The chromatin configuration at the ORF50 promoter is clearly important in regulating KSHV reactivation *in vitro* and *in vivo*. Understanding the sequence of events resulting in the recruitment of

remodelling complexes to the ORF50 promoter probably lies at the heart of a molecular understanding of reactivation.

As well as factors cooperating with RTA, interactions that potentially repress reactivation have been described and are summarised in table 1.1.15.2. The potentially repressive interactions of KSHV LANA are particularly interesting. LANA is able to interfere with RTA-mediated transactivation of the ORF50 promoter (Lan *et al.*, 2004), possibly through interactions with CBP, RBP-Jκ and RTA (Lim *et al.*, 2001; Lan *et al.*, 2005a; Lan *et al.*, 2004). It is tempting to speculate that LANA inhibits the interaction of RTA with CBP and RBP-Jκ. Such negative regulation would prevent undesired reactivation from a 'leaking' ORF50 promoter and ensure that only significant RTA expression would result in autoactivation of the ORF50 promoter and entry into the lytic cycle. Indeed, such negative regulation may be common to many rhadinoviruses. LANA homologues are capable of suppressing productive infection of rhesus monkey rhadinovirus and *Herpesvirus saimiri in vitro* (DeWire and Damania, 2005; Schäfer *et al.*, 2003).

Table 1.1.15.2. Potential negative regulators of RTA.

Protein	Capable of repressing RTA activity?
RBP-Jk	Interacts with LANA (Lan *et al.*, 2005a)
K-bZIP	Interacts with RTA and represses RTA-mediated transactivation of K8 promoter (Liao *et al.*, 2003) Interacts with CBP repressing AP-1 mediated transactivation (Hwang *et al.*, 2001)
LANA	Interacts with RTA (Lan *et al.*, 2004) Interacts with RBP-Jκ (Lan *et al.*, 2005a) Interacts with CBP (Lim *et al.*, 2001)
HDAC1	Interacts with RTA (Gwack *et al.*, 2001)
Nuclear factor kappa B	Inhibits RTA-mediated transactivation (Brown *et al.*, 2003)

1.1.16. Primary effusion lymphoma

Body cavity based lymphomas (BCBL) presenting in the body cavities of AIDS patients were described as early as 1989 (Knowles *et al.*, 1989). Not long after the discovery of KSHV, an association between BCBL and KSHV was reported (Cesarman *et al.*, 1995a). The realisation that not all BCBLs were associated with KSHV led to the

designation of KSHV-positive BCBLs as primary effusion lymphomas (PELs), a distinct clinicopathologic entity (Nador *et al.*, 1996)

PEL accounts for approximately 3% of all HIV-related non-Hodgkin lymphomas (Knowles, 2003) and usually presents as a lymphomatous effusion in the pleural, pericardial or peritoneal cavities, usually without significant tumour mass (reviewed in Ablashi *et al.*, 2002). Although the majority of PEL patients are HIV positive and immunosuppressed (Komanduri *et al.*, 1996), PEL has also been observed in HIV-negative individuals (Nador *et al.*, 1996). The molecular and clinical features of PEL are identical in AIDs-related and non-related PEL. However, AIDS unrelated PEL tends to develop later in a life (Nador *et al.*, 1996; Carbone *et al.*, 1996).

The origin of PEL cells was initially difficult to determine because of the intermediate immunophenotype of these cells (reviewed in Matolcsy, 1999). The lack of classical B-cell associated antigens, most notably cell determinant (CD) 19, expressed by PEL cells resulted in the B-cell origin of these tumours being determined by analysis of immunoglobulin (Ig) gene rearrangements (Nador *et al.*, 1996; Carbone *et al.*, 1996). Most PEL-cells have hypermutated immunoglobulin genes suggesting they have been through a germinal centre reaction (Matolcsy *et al.*, 1998; Fais *et al.*, 1999). In addition, most PEL cells express CD45, CD30, CD38, CD71, epithelial membrane antigen (EMA), the antigen recognised by monoclonal antibody Ki-67 and CD138/syndecan-1. This expression profile is reminiscent of cycling plasmablasts. The surface antigen CD138 is a marker of pre-B-cells and plasma cells and is not expressed by any other lymphomatous effusion (Gaidano *et al.*, 1997), this marker is therefore useful for clinical diagnosis and for defining the stage of B-cell development PEL represents.

The mature immunophenotype and hypermutated immunoglobulin genes suggest that PEL cells represent a preterminal stage of B-cell differentiation, close to that of plasma cells (Gaidano *et al.*, 1997). This hypothesis was subsequently confirmed using gene expression profiling which determined that PEL cells have a plasmablastic gene expression profile (Jenner *et al.*, 2003; Klein *et al.*, 2003). Most PEL cells contain somatically hypermutated Ig genes (reviewed in Matolcsy, 1999) suggesting the tumours developed from post-germinal centre B-cells. However many cases with germline rearranged Ig genes have been described, such as BC-3 (Arvanitakis *et al.*,

1996), suggesting passage through a germinal centre reaction is not essential for the development of PEL.

The association of KSHV with PEL has been confirmed by multiple studies (Pastore *et al.*, 1995; Carbone *et al.*, 1996, Otsuki *et al.*, 1996; Gessain *et al.*, 1997, Karcher and Alkan, 1995) and KSHV is present within every PEL cell (Lennette *et al.*, 1996; Kellam *et al.*, 1997). Approximately 20-200 copies of the viral episome are present in every cell (Gessain *et al.*, 1997; Cesarman *et al.*, 1995a) and KSHV latent gene expression is essential for PEL cell survival (Guasparri *et al.*, 2004). Over three quarters of PEL cells are coinfected with EBV and it has been suggested that EBV coinfection favours tumour development after the germinal centre reaction whereas EBV negative PEL can originate from naïve B-cells as well as post-germinal centre B-cells (Hamoudi *et al.*, 2004). Crucially, it is difficult to distinguish the gene expression profile of EBV positive and EBV negative PELs (Jenner *et al.*, 2003; Klein *et al.*, 2003) suggesting KSHV is the dominant viral factor contributing to PEL (Fan *et al.*, 2005).

The presence of clonal Ig gene rearrangements suggests PEL is a clonal tumour (Nador *et al.*, 1996). EBV and KSHV terminal repeat analysis indicates that γ-herpesvirus episomes are usually clonal in PEL (Judde *et al.*, 2000; Boulanger *et al.*, 2005) indicating that latent infection precedes tumour development or provides a selective advantage to an infected subset of polyclonal tumour cells. PEL is uncommon, even in populations where KSHV is endemic and rare KSHV negative EBV negative tumours similar to PEL have been described (Barbey *et al.*, 1990). KSHV infection can therefore be considered as a significant risk factor for the development of PEL but other transforming events are also required. For example, the B-cell lymphoma-6 (BCL-6) promoter is frequently mutated in PEL (Gaidano *et al.*, 1999) and trisomy 7 and trisomy 12 are common chromosomal abnormalities (Wilson *et al.*, 2002). No cytogenetic abnormality common to all cases of PEL has been identified, underlining the importance of KSHV infection in the development of PEL.

A feature of PEL is the lack of Ig expression. Despite the similarity of PEL cell-lines to plasmablasts, PEL cells do not secrete Ig. Indeed, surface Ig is not expressed by many PEL-cells (Nador *et al.*, 1996). PEL cells express B-lymphocyte induced maturation protein 1 (BLIMP-1) (described in section 1.2.5.4) and the cleaved 50 kDa form of activating transcription factor (ATF)-6 (described in section 1.2.6.2) (Jenner *et al.*,

2003), a transcription factor complement usually sufficient for terminal differentiation into plasma cells. PEL cells have been reported to lack PU.1, a transcription factor involved in Ig light chain expression (Arguello *et al.*, 2003), but it is unclear whether this is solely responsible for the reduced Ig expression in PEL. The lack of secreted Ig suggests that X-box binding protein 1 (XBP-1) is unspliced in PEL, but the splice status is PEL has not been determined.

PEL cells harbour latent KSHV with less than 1% of cells harbouring KSHV in the lytic cycle (Parravicini *et al.*, 2000; Katano *et al.*, 2000). KSHV genes expressed in PEL cells are summarised in table 1.1.13.2 and unlike KS and MCD, KSHV lytic replication seems to be unimportant in the development and pathogenesis of PEL.

1.1.17. Multicentric Castleman's disease

MCD is an unusual lymphoproliferative disorder that is characterised by episodic lymphadenopathy, fever and splenic infiltration (reviewed in Waterston and Bower, 2004). Prior to the discovery of KSHV, an association between KS and MCD had long been suspected (Rywlin *et al.*, 1983). Soon after the discovery of KSHV, KSHV DNA was detected in samples from patients suffering from MCD (Soulier *et al.*, 1995). In addition, KSHV is strongly associated with MCD, is present in nearly all reported AIDs-associated MCD cases and around half of HIV-negative cases (Soulier *et al.*, 1995; Gessain *et al.*, 1996; Luppi *et al.*, 1996; Izuchukwu *et al.*, 2003). Immunohistochemical analysis of MCD patient lymph nodes revealed that KSHV was present in plasmablasts, mainly in the mantle zone of B-cell follicles (Dupin *et al.*, 1999). It rapidly became clear that KSHV-associated MCD involves KSHV-positive plasmablasts, which have a CD20+ CD5- CD23- CD30- CD38- CD45- immunophenotype, and express germ-line immunoglobulin genes which are lambda light chain restricted (Dupin *et al.*, 1999; Dupin *et al.*, 2000 Du *et al.*, 2001). KSHV-positive MCD plasmablasts only express IgM λ whereas surrounding KSHV negative cells are polytypic (Dupin *et al.*, 1999; Du *et al.*, 2001). The cause of lambda restriction in KSHV-associated MCD plasmablasts is currently unclear.

Despite being lambda light chain restricted, analysis of Ig gene rearrangements (Du *et al.*, 2001) and KSHV terminal repeats (Judde *et al.*, 2000) suggests that MCD plasmablasts are polyclonal in origin. Unlike PEL-cells, coinfection with EBV has not been detected and KSHV positive MCD plasmablasts probably result from KSHV

infection of naïve B-cells (Dupin *et al.*, 1999; Du *et al.*, 2001). MCD disease severity is closely associated with KSHV viral load (Oksenhendler *et al.*, 2000) and it is likely that MCD pathogenesis may reflect active KSHV replication in lymphoid tissue (reviewed in Schulz, 2006). Consistent with this hypothesis, KSHV lytic antigens are readily detectable in a minority of KSHV-positive MCD plasmablasts (Parravicini *et al.*, 2000; Katano *et al.*, 2000).

1.1.18. Kaposi's sarcoma

There are four epidemiologically distinct forms of KS: classic, endemic, post-transplant or iatrogenic and AIDS-associated. Classic KS (reviewed in Iscovich *et al.*, 2000) is the form originally described by Moritz Kaposi (Kaposi, 1872) and occurs predominantly in elderly men of Southern European, Arabic or Jewish ancestry. In areas of sub-Saharan Africa a more aggressive form of KS has been characterised which affects lymph nodes, in addition to the extremities, and sometimes occurs in children. This form is described as endemic KS (Ziegler *et al.*, 1996). There is a long-standing association between transplant recipients and KS (Harwood *et al.*, 1979), and these cases are described as post-transplant KS (reviewed in Gotti and Remuzzi, 1997). KS is the most common neoplasm in patients suffering from AIDS and this aggressive disease is described as AIDS-associated KS (Beral *et al.*, 1990).

It is now widely accepted that KSHV causes KS and there are multiple lines of evidence that this is the case: (i) the KSHV genome is invariably detected in each of the four forms of KS (reviewed in Schulz, 1998; 1999). (ii) KSHV DNA, RNA and antigens are readily detectable in KS spindle cells, and endothelial cells, but not in surrounding healthy cells (Boshoff *et al.*, 1995; Dupin *et al.*, 1999; Parravicini *et al.*, 2000). (iii) KSHV viral load is predictive of the progression to KS (Whitby *et al.*, 1995). (iv) KSHV seroconversion in HIV-positive men, who have sex with men, is predictive of KS development. (v) the prophylactic administration of antiherpetic drugs prevents KS development (Robles *et al.*, 1999). (vi) KSHV is monoclonal in advanced lesions suggesting that infection precedes sarcomagenesis (Judde *et al.*, 2000). (vii) Exogenous expression of the viral G-protein coupled receptor in murine endothelial cells, *in vivo*, induces angioproliferative tumours similar to KS (Montaner *et al.*, 2003).

Early stage KS lesions are composed of small irregular endothelial-lined spaces surrounding normal blood vessels. These lesions described as 'patch stage' are

accompanied by an infiltrate of inflammatory cells. As the disease progresses, 'plaque stage' lesions develop. These lesions involve slit-like vascular channels, containing erythrocytes, lined by spindle cells, which penetrate the dermis. Late stage lesions, described as the 'nodular stage', consist of sheets of spindle cells and slit-like vascular spaces. Spindle cells are a defining feature of Kaposi's sarcoma (reviewed in Gessain and Duprez, 2005) and have a variable immunophenotype expressing endothelial, monocyte, fibroblast and smooth muscle markers. This had led to much controversy surrounding the identity of the precursor cells from which spindle cells are derived. Recently, three molecular studies have illuminated this area. Gene expression profiling of KS-lesions identified spindle cells as having a gene expression profile similar to lymphatic endothelial cells (LECs) (Wang *et al.*, 2004a). Infection of blood vessel endothelial cells (BECs) with KSHV *in vitro* led to the reprogramming of BECs to a more LEC-like state (Wang *et al.*, 2004a; Hong *et al.*, 2004b; Carroll *et al.*, 2004). This suggests that KSHV is able to infect LECs and BECs, and that these reprogrammed cells are the precursors of spindle cells.

In early KS lesions, only around 10% of spindle cells are KSHV positive (Dupin *et al.*, 1999). Although KSHV is associated with all KS lesions, it is clear that paracrine events are essential for sarcomagenesis (reviewed in Ensoli *et al.*, 2001) and many inflammatory cytokines, angiogenic factors and chemokines have been detected in KS-lesions (reviewed in Ensoli, 2001). Interestingly, proteins involved in lytic replication and the inflammatory environment resulting from viral replication may be required for this process (reviewed in Hayward, 2003) suggesting that the minority of cells in KS-lesions expressing lytic antigens may drive sarcomagenesis.

Clonality is an important aspect of all malignancies. Studies examining both the clonality of host cells (Gill *et al.*, 1998) and the clonality of viral TRs in spindle cells (Judde *et al.*, 2000) have yielded conflicting results. This is most likely due to contaminating healthy cells affecting the analysis of host-cell clonality. Crucially, monoclonal or oligoclonal patterns of KSHV TRs are more frequently detected in late-stage, nodular KS-lesions (Judde *et al.*, 2000) suggesting that KSHV infection precedes sarcomagenesis and that a predominantly latent KSHV infection drives spindle-cell proliferation.

1.2. B-cell differentiation and gammaherpesviruses

The developmental process which generates plasma cells and memory B-cells can be divided into three stages: (i) the generation of mature immunocompetent B-cells. (ii) The activation of mature B-cells after encountering antigen. (iii) The differentiation of activated B-cells into plasma cells or memory B-cells. Each stage of B-cell development is summarised here in turn. Particular attention is paid to stage iii as the impact of plasma cell differentiation on KSHV reactivation is considered in chapter 6. The multiple pathways of B-cell development are summarised in figure 1.2.1.1

1.2.1. B-cell maturation

An important aspect of the adaptive immune response is the ability to respond to a varied array of different pathogens. B-cells have a central role in this process, having the potential to produce billions of different immunoglobulins.

B-cells are formed in the bone marrow, or foetal liver, from pluripotent haematopoietic stem cells. Commitment to the B-cell lineage is signified by CD45 expression (Allman *et al.*, 1999) and these cells are described as progenitor (pro)-B cells. Pro-B cells proliferate and differentiate within the bone marrow stimulated by factors such as stromal cell-derived factor-1 (SDF-1) (Nagasawa *et al.*, 1996), stem-cell factor (SCF) (McNiece *et al.*, 1991) and interleukin (IL)-7 (reviewed in Lee *et al.*, 1988), which are produced by the surrounding stromal cells. During pro-B cell development CD19, the major cell determinant of B-cells, is expressed. CD19 expression is upregulated by the B-cell-specific activating protein (BSAP) (Kozmik *et al.*, 1992) encoded by the *PAX-5* locus (Adams *et al.*, 1992). Once expressed, BSAP maintains commitment to the B-cell lineage, suppressing alternative lineage choices (Nutt *et al.*, 1999). Interestingly, microRNAs (described in section 1.3) have recently been implicated in B-cell lineage commitment (Chen *et al.*, 2004).

During the pro-B cell stage, rearrangement of the immunoglobulin heavy chain locus occurs (reviewed in Collins *et al.*, 2003; Hozumi and Tonegwa, 1976; Brack *et al.*, 1978). First to occur is the joining of the diverse (D) and joining (J) gene segments of the immunoglobulin (Ig) heavy chain (IgH) (Hardy *et al.*, 1991). This is followed by the recombination of the variable (V) region with the DJ segment to form a complete

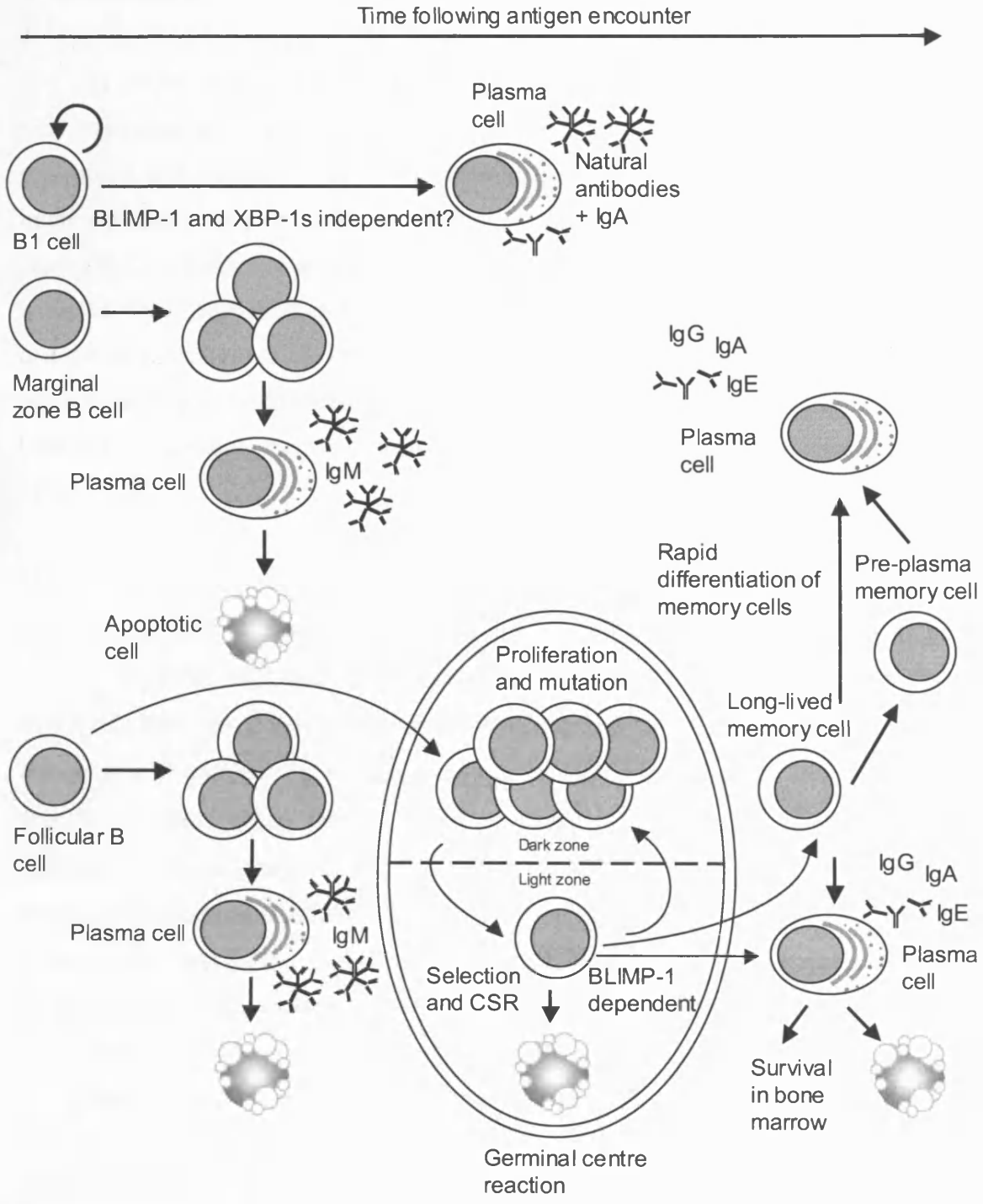


Figure 1.2.1.1. The multiple pathways of B-cell development (Adapted from Shapiro-Shelef and Calame 2005).
A diagrammatic representation of the multiple pathways of B-cell development.

IgH gene. The process of immunoglobulin gene rearrangement is catalysed by recombinase-activating genes (RAG) 1 and 2 (Oettinger *et al.*, 1990) and terminal deoxynucleotide transferase (TdT) (Komori *et al.*, 1993). Successful rearrangement of the IgH locus is a major checkpoint in B-cell maturation and successful IgH rearrangements are positively selected based on their ability to associate with the proteins λ5 and VpreB. These two proteins interact and form a structure similar to an immunoglobulin light chain (Karasuyama *et al.*, 1990), described as the surrogate light chain (SLC) (Cherayil *et al.*, 1991). Cells that fail to generate an IgH which complexes with the SLC, described as the pre-B cell receptor, are unable to complete B-cell maturation (Kitamura *et al.*, 1992). Conversely, cells expressing a functional pre-B-cell receptor undergo a proliferative burst (Hardy *et al.*, 1991) and RAG gene expression is transiently downregulated to prevent further gene rearrangements (Grawunder *et al.*, 1995).

The subsequent pre-B-cell stage is characterised by rearrangement of the genes encoding the Ig light chain (IgL) (reviewed in Ehlich and Kuppers, 1995). There are two loci encoding light chain gene segments, κ and λ. The κ loci are rearranged first, and if this fails, the λ loci are rearranged (Mehr *et al.*, 1999). Successful light chain rearrangement results in the expression of IgL. If the expressed IgL is able to complex with the μ heavy chain, forming IgM, an immature BCR complex is then formed through association with Igα and Igβ (CD79a and b). If the immature BCR signals above a critical threshold, RAG gene expression is, again, downregulated (Grawunder *et al.*, 1995; Lang *et al.*, 1996). If signalling through the BCR is activated by self-antigen in the bone marrow, there are three possible outcomes: (i) high affinity interactions result in apoptosis (clonal deletion) (Nemazee and Burki, 1989) whereas low affinity interactions result in (ii) the maintenance of RAG expression whilst the light chains are replaced (receptor editing) (Tiegs *et al.*, 1993) or (iii) anergy, a state where antigen binding does not trigger intracellular signalling (Goodnow *et al.*, 1989). Mature B-cells, which do not recognise self-antigen, exit the bone marrow and enter circulation.

1.2.2. Minority B-cell subsets

B-cells can be divided into many subsets based on their location, developmental stage and immunophenotype. However, all B-cells, at one stage of differentiation, can be divided into 3 subsets. These are described as mature recirculating/follicular/B-2 B-

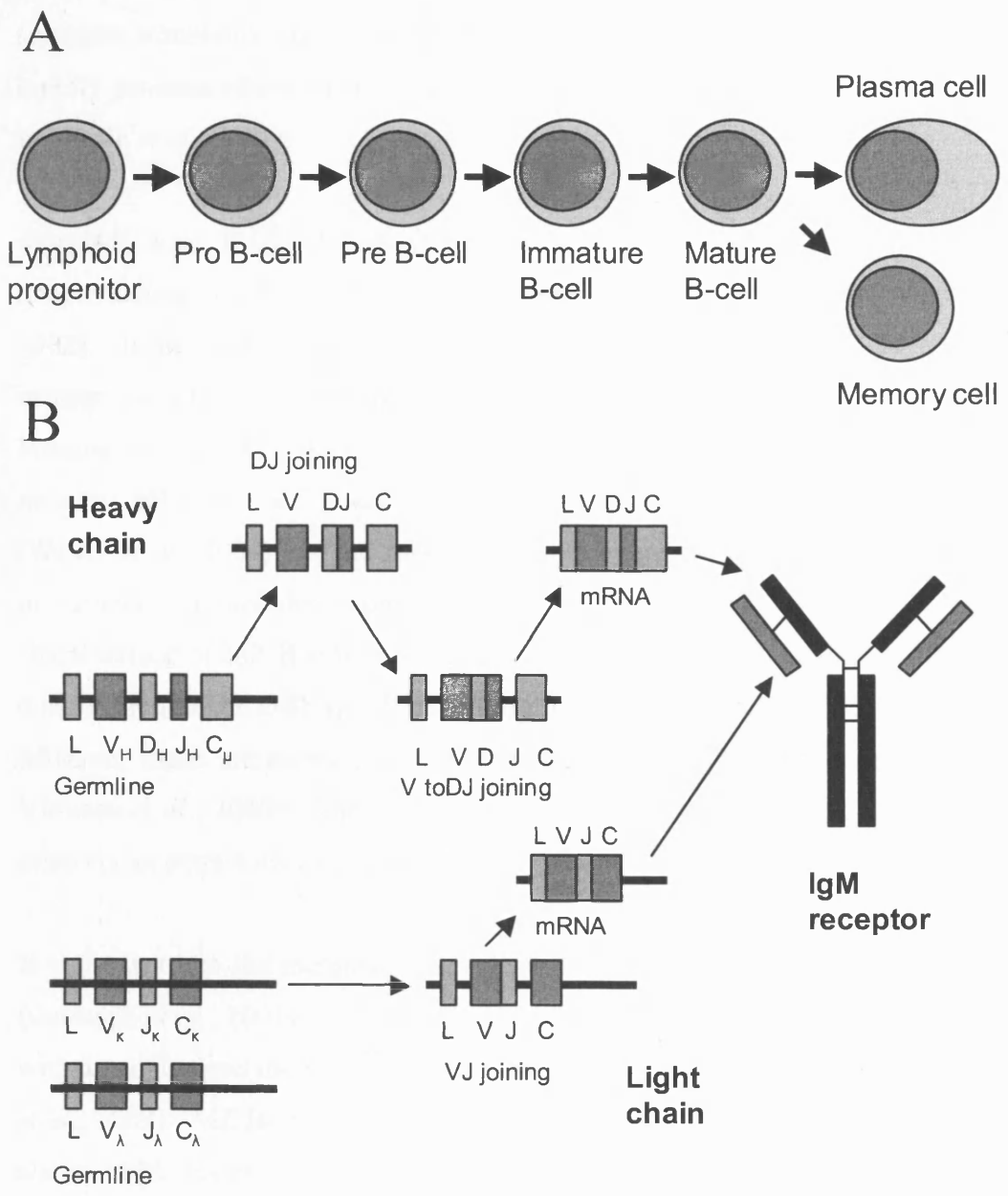


Figure 1.2.1.2. B-cell development and immunoglobulin gene rearrangements (adapted from Ollila and Vilinen, 2005).
A. A diagrammatic representation of the major stages of B-cell development. B. A diagrammatic representation of the sequential gene rearrangements at the immunoglobulin loci. A representation of the structure of the IgM component of the BCR is also shown.

cells, marginal zone B-cells and B-1 B-cells. B-2 B-cells are the most common B-cell subset, and most of the details described in sections 1.2 apply to these cells. Marginal zone and B-1 B-cells make 'natural antibodies', antibodies that arise without any known antigenic stimulation (reviewed in Martin and Kearney, 2000) and have the capacity to rapidly generate effector-plasmablasts in early stages of an immune response (reviewed in Martin *et al.*, 2001).

Marginal zone (MZ) B-cells (reviewed in Pillai *et al.*, 2005b) are named after noncirculating B-cells which reside in the splenic marginal zone of rodents (Gray *et al.*, 1982). In humans however, MZ B-cells circulate in a manner similar to circulating memory cells (reviewed in Spencer *et al.*, 1998). Indeed, the splenic marginal zone, in humans, is populated with both MZ B-cells and memory B-cells. Circulating IgM^+ memory cells and MZ B-cells carry similar somatic mutations in their IgM genes (Weller *et al.*, 2004) and are indistinguishable by gene expression profiling (Weller *et al.*, 2004) and probably represent the same B-cell subset. The somatic mutations characteristic of MZ B-cells also occur in individuals suffering from HyperIgM-1 who, due to a mutated CD40 ligand, cannot form germinal centres (Weller *et al.*, 2001). In addition, these mutations occur in response to T-independent antigens in mice (de Vinuesa *et al.*, 2000). These observations suggest that MZ B-cell somatic mutations arise via an extra-follicular route but the details of this process are unclear.

B-cell entry into the marginal zone subset is favoured by low affinity BCR signalling (Cariappa *et al.*, 2001) and requires notch signalling. This is clearly apparent in mice, with an engineered defect in RBP-Jκ, who are unable to generate MZ B-cells (Tanigaki *et al.*, 2002). MZ B-cells have elevated levels of BLIMP-1 (Martin *et al.*, 2001). This allows MZ B-cells to rapidly differentiate into plasmablasts within hours of encountering antigen. This provides the host with a rapid and substantial IgM response, an essential defence against blood-borne pathogens (Zandvoort *et al.*, 2002).

The B-1 subset of B-cells was originally identified based on the presence of CD5 (Caligaris-Cappio *et al.*, 1982). However it is now clear that CD5- B-1 B-cells also exist (Kantor *et al.*, 1992). Indeed, CD5 is a poor marker of B-1 B-cells because CD5 expression can also be induced on follicular B-cells (Cong *et al.*, 1991). B-1 B-cells are the main population of B-cells in foetal life (Bhat *et al.*, 1992) but are the minority B-cell subset in adult life (Dono *et al.*, 1996). B-1 B-cells are extremely long-lived cells

and were originally thought to exist throughout an individual's life, without a requirement for renewal. Although the details of B-1 B-cell self-renewal have yet to be unravelled, it is clear that adult bone marrow can produce B-1 B-cells (Waddick and Uckun, 1993). BCR activation, through encounter with self-antigen, seems important in B-1 B-cell development (reviewed in Hayakawa *et al.*, 2000), providing a possible explanation for the increased proportion of autoantibodies produced by this subset (Qin *et al.*, 1999).

Similarly to MZ B-cells, B-1 B-cells are involved in the production of 'natural antibody' and isolated B-1 B-cells secrete IgM *in vitro* (Casali *et al.*, 1987). The IgM expressed by B-1 B-cells are not mutated (Kuppers *et al.*, 1993). Interestingly, the transcriptional programme resulting in the production of 'natural antibodies' by B-1 B-cells is different to follicular B-cells, with no apparent requirement for BLIMP-1 or the spliced form of XBP-1 (Tumang *et al.*, 2005).

1.2.3. B-cell activation

Heightened reactivity to antigen recall is the central defining characteristic of the adaptive immune response (Burnet and Fenner, 1949). The follicular B-cell subset can give rise to antigen specific memory B-cells, an essential feature of immunological memory. Follicular B-cells are attracted into follicles of secondary lymphoid organs by B-lymphocyte chemokine (Gunn *et al.*, 1998). In the follicle, B-cells differentiate into IgM^+, IgD^+ mature B-cells. Only a fraction of immature B-cells differentiate into mature B-cells, with self-reactive B-cells suffering clonal deletion (Russell *et al.*, 1991), or failing to compete for entry into the follicular niche (Cyster *et al.*, 1994). Once in the follicles, B-cells receive pro-survival signals such as the tumour necrosis factor (TNF) family B-cell activating factor (BAFF), also described as BLyS (Harless *et al.*, 2001). Mature antigen naïve B-cell survival also requires low-level signalling through the BCR (Turner *et al.*, 1997).

1.2.3.1. Development of effector T-helper cells: Immune synapse I

The T and B-cells in secondary lymphoid organs are dependent on antigen being presented to them by dendritic cells (DCs). DCs can detect conserved motifs on many different pathogens through Toll-like receptors (TLRs) (reviewed in Kawai and Akira, 2005) and receptors which recognise carbohydrates (Reviewed in Figdor *et al.*, 2002). Pattern recognition stimulates DCs to endocytose and process antigen. This leads to

DCs presenting antigen-derived peptides complexed with class II major histocompatibility complex (MHC) molecules (pMHCII) (reviewed in Quah and O'Neil, 2005). In addition, DC activation through pattern recognition also causes the upregulation of receptors, such as the CC chemokine receptor 7 (CCR-7) (Dieu *et al.*, 1998) or osteopontin receptors (Weiss *et al.*, 2001), which cause DCs to migrate from the site of antigen-encounter to the secondary lymphoid organs.

Once in the secondary lymphoid organ, antigen is presented by dendritic cells in the T-cell zones of lymph nodes or periarteriolar lymphoid sheath (PALS) of the spleen. Antigen specific CD4-positive T-cells interact with DCs forming an immunological synapse (Monks *et al.*, 1998; Grakoui *et al.*, 1999). The main interaction at this immunological synapse is between pMHCII and antigen specific T-cell receptors (TCRs) with prolonged interaction sufficient to drive T-helper (T_H) cell proliferation (Lee *et al.*, 2002b). The coupling of DCs, activated by pattern recognition in peripheral sites, with antigen specific T-cells provides a bridge between the innate and adaptive arms of the immune response.

1.2.3.2. Activation of naïve antigen specific B-cells: Immune synapse II

To activate resting, naïve, antigen-specific B-cells, at least two sets of signals are required (Lanzavecchia, 1985). The naïve B-cell first encounters antigen, usually presented by antigen presenting cells, which activates signalling through the BCR (signal 1). BCR activation also stimulates internalisation of antigen (Batista *et al.*, 2001) which is then processed and presented as pMHCII (Lanzavecchia, 1985). This complex is recognised by the TCRs of clonally expanded T_H cells generated by immune synapse I (signal 2). Although these two signalling events are the main protagonists in B-cell activation, it is clear that a number of costimulatory signals modulate B-cell activation (reviewed in Bishop and Hostager, 2001). CD40 and CD40 ligand have an essential role in class switch recombination (CSR) and germinal centre (GC) formation (reviewed in Banchereau *et al.*, 1994). OX40 (CD134) and OX40-ligand also seem essential for CSR (Murata *et al.*, 2000) and CD27-CD70 interactions may promote plasma cell development (Jacquot *et al.*, 1997). Once activated, B-cells are described as lymphoblasts and proliferate to form a primary focus (Jacob *et al.*, 1991a). At this stage, lymphoblasts can either form a secondary follicle or can differentiate into short-lived plasma cells (Ho *et al.*, 1986) which secrete germline encoded antigen-specific

antibodies (Jacob and Kelsoe, 1992). Exactly how the signals at the T-B-cell synapse interact to specify different cell fates following B-cell activation remains unclear.

Class switch recombination (CSR) (reviewed in Stavnezer and Schrader, 2006) allows the immunoglobulin isotype expressed by a B-cell to be changed. The different IgH constant regions are arranged sequentially in the human genome and CSR involves recombination between switch-region sequences located upstream of each of the IgH constant regions (except ε) resulting in intrachromasomal deletion of the intervening sequence. This results in a different IgH gene being expressed with the variable region. The molecular machinery involved had not been fully defined but involves activation induced cytidine deaminase (AID) (Okazaki *et al.*, 2002; Ta *et al.*, 2003) and uracil-N-glycosidase (Rada *et al.*, 2002).

Although the majority of CSR occurs within the germinal centre, the outcome of CSR may be determined early during immune synapse II. CD40-CD40L interactions are important for CSR (reviewed in Banchereau *et al.*, 1994), a function which may be substituted by BAFF expression (Litinskiy *et al.*, 2002). Despite the importance of these interactions, the extent of CSR seems to be determined by T_H-cell cytokine release with IL-4 (Kuhn *et al.*, 1994), IL-21 (Ozaki *et al.*, 2002), IFN-γ (Snapper and Paul, 1987), transforming growth factor β (Coffman *et al.*, 1989) and BSAP (Qiu *et al.*, 1998) all implicated in this process. Additionally, there may be a role for IL-2, IL-5 and IL-6 (Kopf *et al.*, 1994) in supporting the survival of B-cells expressing different classes of immunoglobulin.

1.2.3.3. The germinal centre reaction

Lymphoblasts can migrate into a primary follicle, forming a secondary follicle. Lymphoblasts expressing BCRs with high affinity for antigen are preferentially recruited at this stage (Shih *et al.*, 2002). The proliferating lymphoblasts displace resting follicular B-cells generating a germinal centre (reviewed in Wolniak *et al.*, 2004; McHeyzer-Williams, 2003; Guzman-Rojas, 2002). A GC reaction is defined anatomically by the formation of a zone of proliferating centroblasts, described as the dark zone, and a zone of noncycling centrocytes, described as the light zone. The germinal centre is a cyclical reaction in which B-cell clonal expansion is followed by BCR diversification and affinity-selection of BCRs. This results in affinity maturation, a process which generates BCRs with increased affinity for antigen.

Once in the germinal centre, clonal expansion is coupled to somatic hypermutation (Jacob *et al.*, 1991b; Berek *et al.*, 1991). Somatic hypermutation results in the introduction of substitutions, and rare insertions and deletions, within the variable regions of Ig genes. AID is an essential enzyme involved in SHM (Revy *et al.*, 2000; Muramatsu *et al.*, 2000) although its exact mode of action is unclear (reviewed in Franklin and Blanden, 2004; Honjo *et al.*, 2005), it is clear that dsDNA breaks (Bross *et al.*, 2000) and error prone DNA polymerases are involved (Zan *et al.*, 2001). Approximately one mutation is inserted per cell division (McKean *et al.*, 1984) and the near-random mutation of variable regions creates BCRs with increased or decreased affinity for antigen. GC B-cells with high affinity antigen receptors out-compete those with lower affinity receptors, in the light zone, through receiving survival signals through their BCR (Liu *et al.*, 1989). Cells expressing BCRs with lowered affinity for antigen are deleted through apoptosis (Takahashi *et al.*, 1999). The exact method of antigen presentation and selection in the germinal centre is unclear, but must involve antigen free of immune complexes (Hannum *et al.*, 2000) and probably involves follicular DCs (FDCs) (reviewed in Kosco-Vilbois, 2003) and antigen specific T-cell help (Kim *et al.*, 2001).

Following positive selection in the light zone, centrocytes can return to the dark zone and undergo another round of clonal expansion and SHM. In this way higher affinity mutants of the original BCR are generated through cyclical rounds of mutation and selection. A process described as affinity maturation. Eventually, B-cells which survive the GC reaction leave the germinal centre and differentiate into memory or plasma cells. The cues which cause B-cells to exit the germinal centre are unknown. Interestingly, B-cells deficient for AID (Muramatsu *et al.*, 2000) and BLIMP-1 (Shapiro-Shelef *et al.*, 2003) accumulate in the GC suggesting that intrinsic B-cell mechanisms may be involved. In addition, CD40 signalling is able to downregulate B-cell lymphoma (BCL)-6 (described in section 1.2.5) expression (Panagopoulos *et al.*, 2004), which de-represses BLIMP-1 expression, suggesting a role for T-cell help in this process.

1.2.4. Memory B-cells

Exit from the germinal centre results in at least two different B-cell fates, memory cells and plasma cells. Memory B-cells are, CD27 positive (Klein *et al* 1998), long-lived

nondividing cells (Maruyama *et al.*, 2000) which circulate in the blood (Benner *et al.*, 1977) or colonise the MZ of the spleen (Liu *et al.*, 1988). Upon secondary challenge with antigen, memory cells rapidly differentiate into antibody-secreting plasma cells, (Burnet and Fenner, 1949; Mitchell *et al.*, 1972; Arpin *et al.*, 1997) providing a rapid and specific immune response. Interestingly, in addition to antigen, memory B-cells seem poised to respond to polyclonal activation through pattern recognition and T-cell help (Bernasconi *et al.*, 2002; 2003), whereas naïve B-cells are not responsive to polyclonal signals (Bernasconi *et al.*, 2003). This facilitates the persistent production of antigen specific antibodies, and maintenance of the memory pool, in the absence of specific antigen.

The memory compartment includes post-GC, B-cell-CD45 positive (Bleesing *et al.*, 2003), memory cells, the best characterised B-cell memory compartment (Mitchell *et al.*, 1972). More recently, a second B-cell-CD45 negative memory compartment, described as the pre-plasma memory compartment, has been defined (McHeyzer-Williams *et al.*, 2000; Driver *et al.*, 2001). The specific functions of this subset are currently unclear (Blink *et al.*, 2005). In addition, other minority post-GC memory subsets may exist (Cascalho *et al.*, 2000).

The observation that B-cells unable to express BLIMP-1 can develop post-GC memory cells but not pre-plasma memory cells (Shapiro-Shelef *et al.*, 2003) led McHeyzer Williams and colleagues to suggest a linear model of memory B-cell development (depicted in figure 1.2.1.1) in which post-GC memory B-cells lie upstream of pre-plasma memory cells (Shapiro-Shelef *et al.*, 2003; reviewed in McHeyzer-Williams and McHeyzer-Williams, 2005). However, this model has yet to be verified and the cues which determine the post-GC cell fate are largely unknown.

1.2.5. Plasma cells

Plasma cells are the effector cells of the B-cell lineage (reviewed in Calame *et al.*, 2003; Lin *et al.*, 2003b; Shapiro-Shelef and Calame, 2004; Shapiro-Shelef and Calame, 2005). Plasma cells are terminally differentiated B-cells which are able to synthesise and secrete large amounts of immunoglobulins, an integral feature of the innate and adaptive immune responses.

The signals resulting in commitment to plasma cell differentiation are not yet understood. However, many signals have been implicated in this process. Yet again, signals from the BCR seem important in this process. BCR signalling can result in phosphorylation of B-cell lymphoma-6 (BCL-6) resulting in degradation via the ubiquitin/proteasome pathway *in vitro* (Niu *et al.*, 1998). Crucially, the activation of BCR signalling and subsequent degradation of BCL-6 is not sufficient to cause BLIMP-1 expression or plasma cell differentiation (Schliephake and Schimpl, 1996). As well as the BCR-antigen interaction, other signals are required. Indeed, activation of B-cell signalling in the presence of IL-2 and IL-5 does result in BLIMP-1 expression and differentiation into plasma cells (Schliephake and Schimpl, 1996). In addition, IL-6 (Piskurich *et al.*, 2000), IL-10 (Choe and Choi, 1998) and IL-21 (Ozaki *et al.*, 2004) have also been implicated in plasma cell development. Although these cytokines activate many different signalling pathways, the signal transducer and activator of transcription (STAT) family of transcription factors, particularly STAT-3 are thought to be involved in the upregulation of BLIMP-1 expression (Reljic *et al.*, 2000). Interestingly, at each cell division during clonal expansion in the GC reaction there is a predictable probability of commitment to the plasma cell fate (reviewed in Tangye and Hodgkin, 2004). This probability is further increased in the presence of IL-4 or IL-5 (Hasbold *et al.*, 2004) which suggests that intrinsic and extrinsic mechanisms influence the commitment to plasma cell differentiation.

Ultimately, any signals which promote plasma cell differentiation manifest themselves in changes in the transcription factor repertoire. In order for plasma cell differentiation to occur, the transcription factors required for the maintenance of the pre-plasma B-cell phenotype must be repressed and transcription factors inducing differentiation must be activated. Many of the transcription factors involved in defining the B-cell/GC state and those governing plasma cell differentiation are well defined and summarised in figure 1.2.5. And the impact of each transcription factor is considered in turn.

1.2.5.1. *PAX-5*/BSAP

The BSAP protein encoded by the *pax-5* locus is a helix-turn-helix DNA binding protein that is required for commitment and maintenance of the B-cell fate (Nutt *et al.*, 1999). BSAP expression is restricted to B-cells, in cells of a haematopoietic lineage, and is involved in isotype switching (Qiu *et al.*, 1998). BSAP acts as a transcriptional repressor, blocking differentiation into plasma cells. This is achieved through multiple

activities which converge on the transcription factor XBP-1. BSAP is able to directly repress XBP-1 transcription (Reimold *et al.*, 1996). In addition, BSAP is able to repress immunoglobulin expression through repressing J-chain (Rinkenberger *et al.*, 1996), IgH expression (Singh *et al.*, 1993; 1996) and kappa light chain expression (Shaffer *et al.*, 1997). Immunoglobulin expression is thought to be the driving force behind the expression of the spliced form of XBP-1 (described in section 1.2.6.2) and BSAP therefore reduces XBP-1 expression and XBP-1 splicing.

1.2.5.2. BCL-6 and metastasis-associated 1 family member 3 (MTA3)

BCL-6 is a sequence specific transcriptional repressor belonging to the poxvirus and zinc finger (POZ)/Zinc-finger protein family (Chang *et al.*, 1996) and is expressed at high levels in GC B-cells (Cattoretti *et al.*, 1995). Crucially, BCL-6 is required for GC formation and BCL-6 knockout mice are unable to form germinal centres (Dent *et al.*, 1997). BCL-6 expression prevents B-cell exit from the GC and subsequent differentiation into plasma cells. This is achieved through downregulating BLIMP-1 expression (Shaffer *et al.*, 2000; Tunyaplin *et al.*, 2004). Consistent with this, plasma cell differentiation is accelerated *in vitro* and serum antibody levels are increased in BCL-6 knockout mice (Tunyaplin *et al.*, 2004). BCL-6 is able to suppress BLIMP-1 expression through at least two different mechanisms. BCL-6 binds to intronic sequences within the positive-regulatory domain containing 1 (*prdm1*) gene, which encodes BLIMP-1, repressing BLIMP-1 expression (Tunyaplin *et al.*, 2004). In addition, BCL-6 is able to interfere with STAT-3 signal transduction, preventing the action of cytokines favouring plasma cell differentiation (Reljic *et al.*, 2000).

Interestingly, metastasis-associated 1 family member 3 (MTA3) is required for BCL-6-mediated repression of BLIMP-1 expression. Ablation of MTA3 expression using RNAi has little affect on BCL-6 expression, but results in the de-repression of BLIMP-1 expression and subsequent plasma cell differentiation. Astonishingly, exogenous expression of BCL-6 in the plasma cell-like multiple myeloma cell line NCI-H929, results in MTA3 dependent de-differentiation. These cells lose BLIMP-1 expression and resume expression of genes characteristic of GC B-cells, such as CD19 (Fujita *et al.*, 2004). MAP kinase signalling (Niu *et al.*, 1998) and BCL-6 acetylation by CBP (Bereshchenko *et al.*, 2002) have both been implicated in the negative regulation of BCL-6. The sequence of events resulting in BCL-6 inactivation and degradation *in vivo*

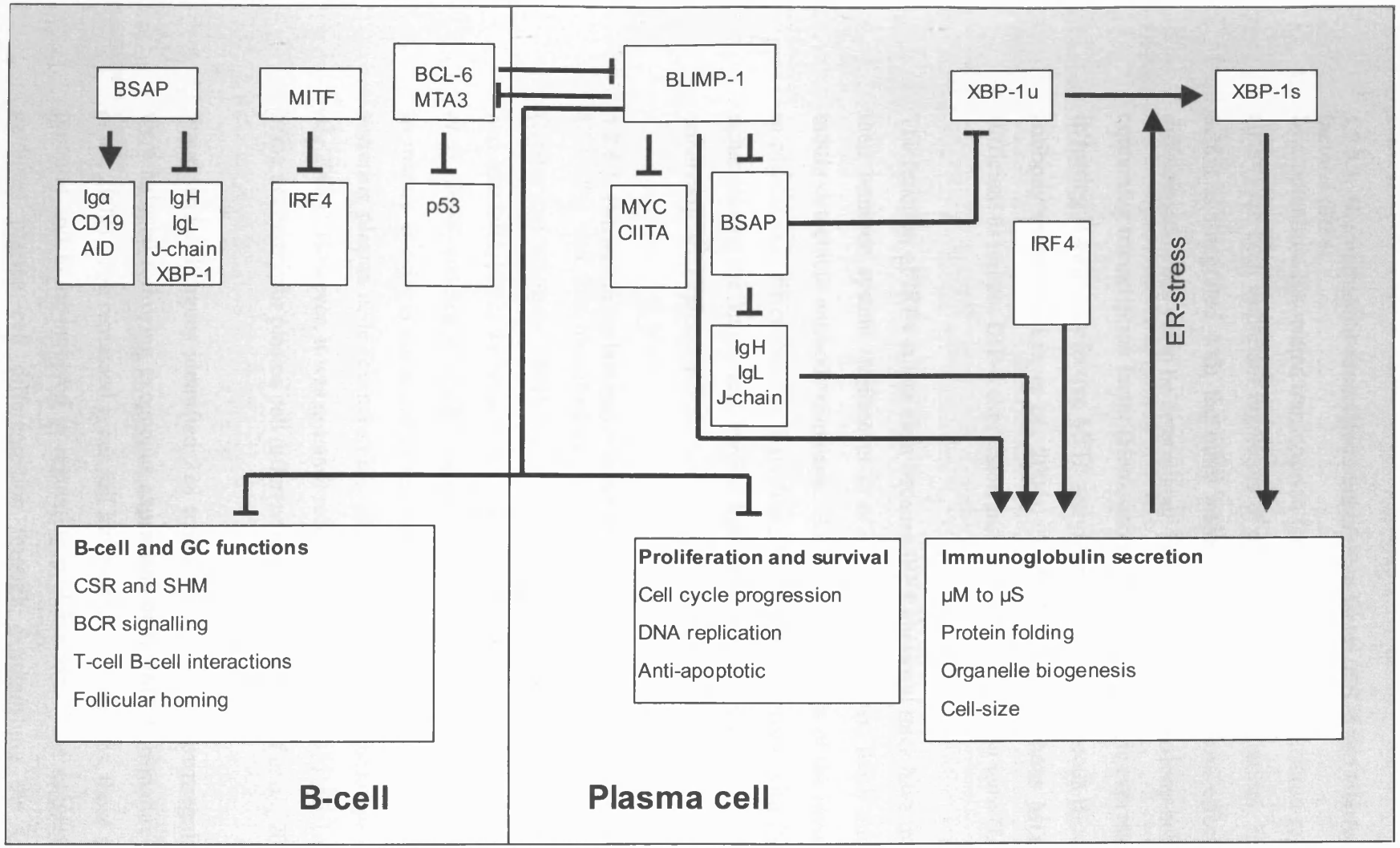


Figure 1.2.5 The transcription factors involved in plasma cell development (adapted from Shapiro-Shelef and Calame, 2005)
A diagrammatic representation of the transcription factors involved in plasma cell development ant their affect on B-cell and plasma cell gene expression programmes.

are currently unclear but probably involve BCR signalling (Niu *et al.*, 1998), T-cell help (Panagopoulos *et al.*, 2004) and cytokine signalling (Schliephake and Schimpl, 1996).

1.2.5.3. Micropthalmia-associated transcription factor (MITF) and Interferon-regulatory factor-4 (IRF4)

Micropthalmia-associated transcription factor (MITF) and interferon-regulatory factor-4 (IRF4) are both important regulators of plasma cell differentiation. Exactly how their action is integrated with the other major transcription factors effecting plasma cell differentiation has yet to be determined. MITF is a basic helix-loop-helix leucine zipper containing transcription factor (Hemesath *et al.*, 1994) which is expressed at high levels in resting B-cells. Defective MITF activity results in spontaneous B-cell activation and antibody secretion (Lin *et al.*, 2004). Furthermore, exogenous MITF expression is sufficient to repress IRF-4 expression and antibody secretion *in vitro* (Lin *et al.*, 2004).

The function of IRF4 is less clear because IRF4 knockout mice have multiple defects in their immune system (Mittrucker *et al.*, 1997). However, these mice are unable to mount detectable antibody responses. Although the details of the involvement of IRF4 in plasma cell differentiation is unclear, IRF4 heterodimerises with PU.1 and binds to enhancers of the kappa and lambda light chains (Eisenbeis *et al.*, 1995), potentially enhancing IgL expression.

1.2.5.4. B-lymphocyte induced maturation protein 1 (BLIMP-1)

BLIMP-1 was first identified as a novel repressor of beta-interferon gene expression (Keller and Maniatis, 1991) and is a five zinc-finger motif containing protein (Keller and Maniatis, 1992) which acts as a transcriptional repressor by recruiting HDACs (Yu *et al.*, 2000) and histone methyltransferases (Gyory *et al.*, 2004). BLIMP-1 expression in mature B-cells is sufficient to induce terminal differentiation into immunoglobulin secreting plasma cells (Turner *et al.*, 1994; Schliephake and Schimpl, 1996; Shaffer *et al.*, 2002). However, it was not until relatively recently that BLIMP-1 was identified as being necessary for plasma cell differentiation (Shapiro-Shelef *et al.*, 2003).

Staudt and colleagues identified 228 transcripts that were downregulated and 32 that were induced following exogenous expression of BLIMP-1 in mature B-cells (Shaffer *et al.*, 2002). The repressed genes fell into two main categories, those specifying B-cell function and those involved in regulating proliferation. This suggests that BLIMP-1 mediates plasma cell differentiation through extinguishing the GC B-cell gene

expression programme and de-repressing genes required for plasma cell differentiation. Crucially, BLIMP-1 represses both BCL-6 expression (Shaffer *et al.*, 2002) and Pax-5 expression (Lin *et al.*, 2002). BCL-6 repression de-represses BLIMP-1 expression (Tunyaplin *et al.*, 2004) amplifying the induction of BLIMP-1 expression. PAX-5 repression de-represses XBP-1 (Reimold *et al.*, 1996), J-chain (Rinkenberger *et al.*, 1996), IgH (Singh *et al.*, 1993; 1996) and kappa light chain expression (Shaffer *et al.*, 1997) which promotes plasma cell differentiation.

1.2.6. Plasma cell differentiation, XBP-1 and the unfolded protein response

The transcription factor X-box binding protein 1 (XBP-1) is a bZIP transcription factor first identified by its ability to bind to the x-box, a conserved transcriptional element, in the human leukocyte antigen (HLA) DR alpha promoter (Liou *et al.*, 1990). XBP-1 is essential for plasma cell differentiation (Reimold *et al.*, 2001). Furthermore, differentiation requires not only the expression of XBP-1 but the expression of the spliced isoform of XBP-1s. The de-repression of XBP-1 and the splicing of XBP-1 under conditions of ER-stress are described here in turn.

1.2.6.1. De-repression of XBP-1 expression

XBP-1 is ubiquitously expressed in mammalian cells. However, XBP-1 expression is substantially repressed by BSAP in B-cells (Reimold *et al.*, 1996). BLIMP-1 expression decreases the abundance of BSAP, which de-represses XBP-1 expression (Reimold *et al.*, 1996; Shaffer *et al.*, 2002). In addition, XBP-1 expression is upregulated by IL-4 (Iwakoshi *et al.*, 2003) and the processed 50 kDa form of ATF-6 (Yoshida *et al.*, 2000).

1.2.6.2. XBP-1s and the unfolded protein response (UPR)

The unfolded protein response (UPR) (reviewed in Schröder and Kaufman, 2005) was first characterised as a stress response resulting in the induction of glucose-regulated proteins (Kozutsumi *et al.*, 1988). In the presence of unfolded and misfolded proteins the UPR regulates the expression of chaperones and foldases, the expansion of the secretory apparatus, the inhibition of protein synthesis, inhibition of cell cycle progression and promotes apoptosis. Using these mechanisms, the UPR is able to minimise the impact of unfolded/misfolded proteins by increasing a cell's capacity to correctly fold proteins and ensuring the removal of cells, unable to achieve this, through apoptosis.

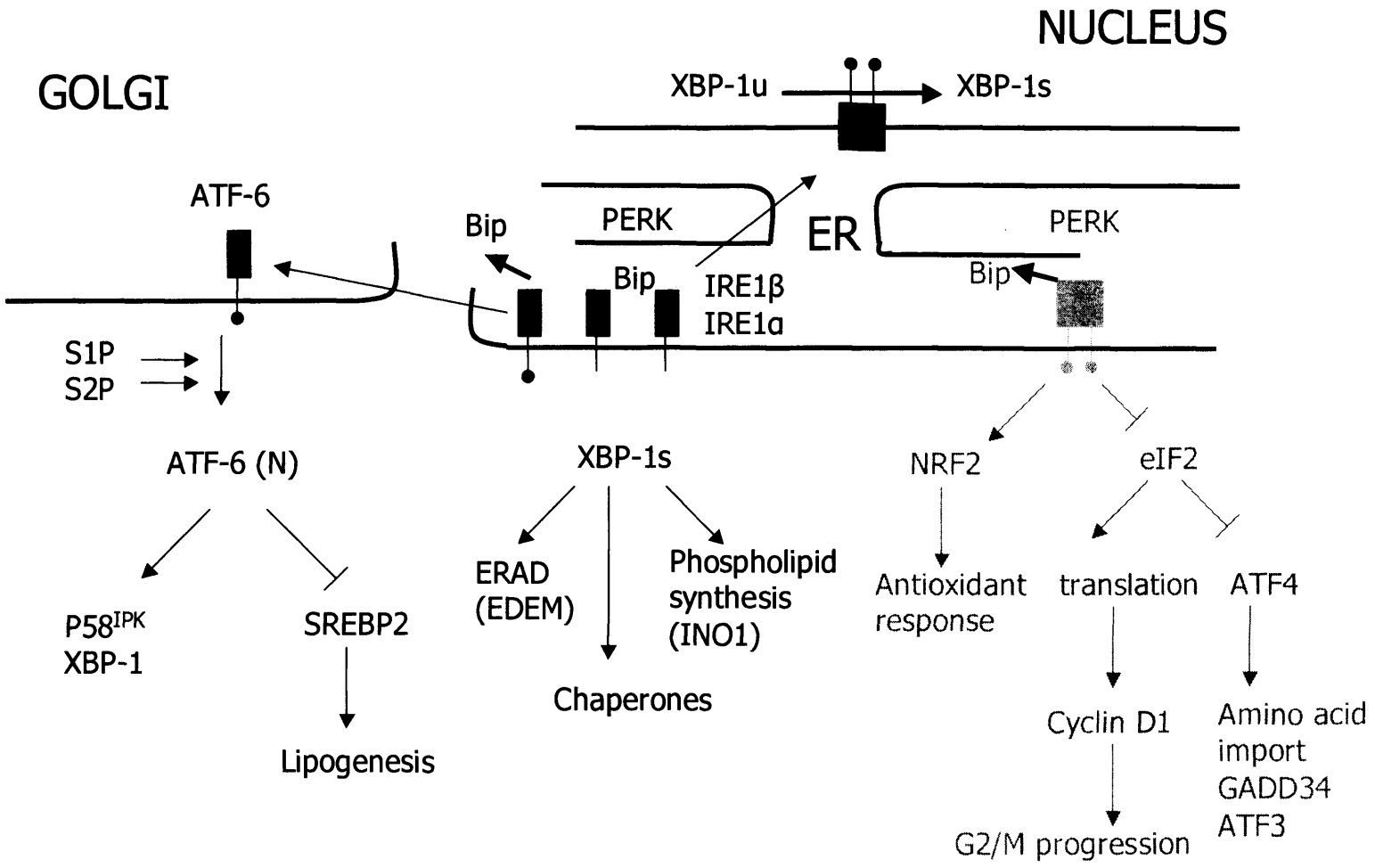


Figure 1.2.5.2 Signalling in the UPR (Adapted from Schröder and Kaufman, 2005)
A diagrammatic representation of signalling pathways in the UPR. The PERK pathway is highlighted in grey due to its apparent absence from the 'physiological UPR'.

The sensing of unfolded proteins in the ER is mediated through the IgH-binding protein BiP (Haas and Wabl, 1983). BiP is able to bind, with low affinity, to short hydrophobic motifs which usually reside in the interior of soluble proteins (Flynn *et al.*, 1991; Blond-Elguindi *et al.*, 1993). Unfolded proteins are therefore able to recruit BiP, which removes BiP from its 'normal' cellular binding partners. Removal of BiP from PKR-like ER kinase (PERK), inositol requiring 1 (IRE1) or activating transcription factor 6 (ATF-6) allows the unfolded protein signal to be transduced across the ER-membrane and these three pathways are summarised in figure 1.2.5.2.

Following the titration of BiP from PERK, PERK undergoes oligomerisation and phosphorylates its substrates. The major substrates of PERK are eukaryotic initiation factor-2alpha (eIF2α) (Harding *et al.*, 1999) and the bZIP transcription factor nuclear factor erythroid 2 (NF-E2)-related factor 2 (NRF2) (Cullinan *et al.*, 2003). Phosphorylation of eIF2α results in a near global inhibition of translation (Harding *et al.*, 1999), which results in decreased levels of high-turnover proteins such as cyclin D1. The removal of cyclin D1 causes cells experiencing ER-stress to arrest in G_1 of the cell cycle (Tomida *et al.*, 1996). Phosphorylated NRF2 migrates from inactive cytosolic complexes to the nucleus, where it upregulates the expression of genes containing antioxidant response elements (Venugopal and Jaiswal, 1998).

The arrest in translation caused by phosphorylated eIF2α is incomplete and the translation of ATF-4 is actually enhanced following the phosphorylation of eIF2α (Harding *et al.*, 2000). The increased abundance of ATF-4 results in increased transcription of ATF-4 target genes. One such gene is GADD34, a protein that is able to accelerate the dephosphorylation of eIF2α, mediated by protein phosphatase 1 (Novoa *et al.*, 2003). GADD34 therefore participates in a negative feedback loop which minimises the inhibition of translation caused by PERK activation. Interestingly, no role for PERK signalling has been reported in plasma cell differentiation. Indeed, the selective activation of different UPR pathways lies at the heart of the 'physiological UPR'. Exactly how IRE1 and ATF-6 are specifically activated without activating PERK during plasma cell differentiation remains unclear.

Following the titration of BiP from ATF-6, two Golgi localisation motifs are exposed on ATF-6 (Shen *et al.*, 2002) causing it to migrate to the Golgi. In the Golgi, two rounds of proteolysis result in the liberation and nuclear translocation of the 50 kDa,

transcriptionally active, N-terminal portion of ATF-6 (Ye *et al.*, 2000). ATF-6 is a bZIP transcription factor able to homodimerise or heterodimerise with other bZIP proteins including XBP-1 (Newman and Keating, 2003). ATF-6 is able to upregulate the expression of many foldases and chaperones and also represses the BiP promoter (Thuerauf *et al.*, 2004). Interestingly, ATF-6 is also involved in negative feedback regulation of the PERK response. ATF-6 upregulates the expression of p58 inhibitor of PKR (IPK) (Yan *et al.*, 2002; van Huizen *et al.*, 2003), another factor capable of promoting the dephosphorylation of eIF2α.

Interestingly, ATF-6 is able to sense ER-stress through an alternative mechanism. ATF-6 has three glycosylation sites, which when glycosylated promote interaction with calreticulin (Hong *et al.*, 2004a). This interaction anchors ATF-6 in the ER and underglycosylated ATF-6 traffics to the Golgi (Hong *et al.*, 2004a). The selective activation of ATF-6 in response to glycosylation provides a link between the calnexin/calreticulin quality control cycle of the ER (reviewed in Kleizen and Braakman, 2004) and the UPR. The selective activation of ATF-6 by this pathway may allow the UPR to be tailored to particular stresses.

Following the removal of BiP from IRE1, IRE1 is activated in manner analogous to PERK. Indeed, the oligomerisation domains of PERK and IRE1 are functionally interchangeable in *S. cerevisiae* (Bertolotti *et al.*, 2000; Liu *et al.*, 2000). Following the dissociation of BiP, IRE1 oligomerises and activates its ribonuclease domain through autophosphorylation (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). Because the lumen of the ER is continuous with the perinuclear space, the activated ribonuclease domains can penetrate the inner leaflet of the nuclear envelope (Lee *et al.*, 2002a). Within the nucleus, activated IRE1 catalyses the excision of a 26 nucleotide unconventional intron (Calfon *et al.*, 2002), from XBP-1 mRNA, in a manner mechanistically similar to pre-tRNA splicing (Gonzalez *et al.*, 1999). Removal of this intron causes a frame shift in the XBP-1 coding sequence resulting in the translation of a 371 amino acid, 54 kDa, XBP-1s isoform rather than the 261 amino acid, 33 kDa, XBP-1u isoform (Calfon *et al.*, 2002).

XBP-1 is a bZIP transcription factor whose isoforms are both capable of binding at least two well-defined sequence elements (Clauss *et al.*, 1996). The extended C-terminal domain can act as a transcriptional activation domain and it has been proposed that

XBP-1u may act as a dominant negative inhibitor of XBP-1s, by occupying target sequences without promoting transcription (Lee *et al.*, 2003a). Consistent with this, proteosomal degradation of XBP-1u seems to be required for full XBP-1s activity (Lee *et al.*, 2003a).

For a bZIP transcription factor, XBP-1 has surprisingly few binding partners (Newman and Keating, 2003). Indeed, XBP-1u is only known to heterodimerise with ATF-6. However, most studies concerning XBP-1 binding-partners were performed using the XBP-1u isoform and it is possible that XBP-1s has an extended repertoire of binding partners.

XBP-1s upregulates a number of target genes associated with the UPR (Lee *et al.*, 2003b) most of which seem to act within the ER. There is a considerable overlap between the target genes of ATF-6 and XBP-1s suggesting that XBP-1 and ATF-6 may serve partially redundant functions (Lee *et al.*, 2003b). Interestingly, one such redundant target is p53 IPK, representing another negative feedback mechanism for the regulation of PERK.

1.2.6.3. The affect of XBP-1s on plasma cell differentiation

XBP-1 is required to make functional plasma cells (Reimold *et al.*, 2001) and the requirement for XBP-1 is downstream of BLIMP-1 expression. Crucially, exogenous expression of XBP-1s in *prdm-1* knockout B-cells does not restore CD138 expression and IgM secretion whereas exogenous expression of BLIMP-1 does (Shapiro-Shelef *et al.*, 2003). In addition, microarray analysis of plasma cell differentiation *in vitro* indicated that 34 of the 36 genes upregulated by XBP-1s were also upregulated by BLIMP-1 expression (Shaffer *et al.*, 2004). Despite this, XBP-1s expression expanded the endoplasmic reticulum, increased cell size, lysosome content, mitochondrial mass, ribosome content and total protein synthesis (Shaffer *et al.*, 2004). This suggests that the role of XBP-1 in plasma cell development is to facilitate development of the professional secretory phenotype, which is required to synthesise and secrete large quantities of immunoglobulin. This model proposes that the de-repression of immunoglobulin expression, caused by BLIMP-1 expression, causes ER-stress which activates IRE1. The subsequent expression of XBP-1s facilitates the final expansion of the secretory apparatus required for the substantial synthesis and secretion of immunoglobulin required in an immune response (Tirosh *et al.*, 2005a).

In an interesting twist, van Anken and colleagues have reported that expansion of the secretory apparatus precedes upregulation of IgM expression (van Anken *et al.*, 2003). This suggests that additional, as yet unidentified, mechanisms may exist to prepare plasma cells for the secretory phenotype.

1.2.7. EBV and B-cell activation and differentiation

The relationship between EBV and human B-cells has been extensively characterised and how EBV manipulates B-cell development is increasingly well understood (Reviewed in Kieff and Rickinson, 2001; Thorley-Lawson, 2001; Thorley-Lawson and Gross, 2004; Thorley-Lawson, 2005). In the absence of a model for KSHV infection of human B-cells, it is useful to consider the interaction between EBV and B-cells to identify possible similarities between the viral lifecycles.

EBV achieves lifelong persistence by establishing a lifelong latent infection in memory B-cells (Babcock *et al.*, 1998; Babcock *et al.*, 1999; Souza *et al.*, 2005). Memory B-cells are not the *in vivo* target for EBV infection. Instead, EBV mimics many aspects of physiological B-cell differentiation and infects naïve B-cells and drives them towards the memory compartment (Thorley-Lawson and Babcock, 1999). This model is summarised in figure 1.2.7.

1.2.7.1. EBV and B-cell activation

During the EBV lifecycle, EBV is able to adopt multiple, latent, gene expression programmes to manipulate differentiation (summarised in table 1.2.7.). Following salivary transmission, EBV is able to infect IgD-positive antigen naïve B-cells present in the nasopharyngeal lymphoid tissue (Joseph *et al.*, 2000). Subsequently, BSAP expressed by the naïve cells activates the expression of Epstein-Barr nuclear antigen (EBNA)-2 (Tierney *et al.*, 2000).

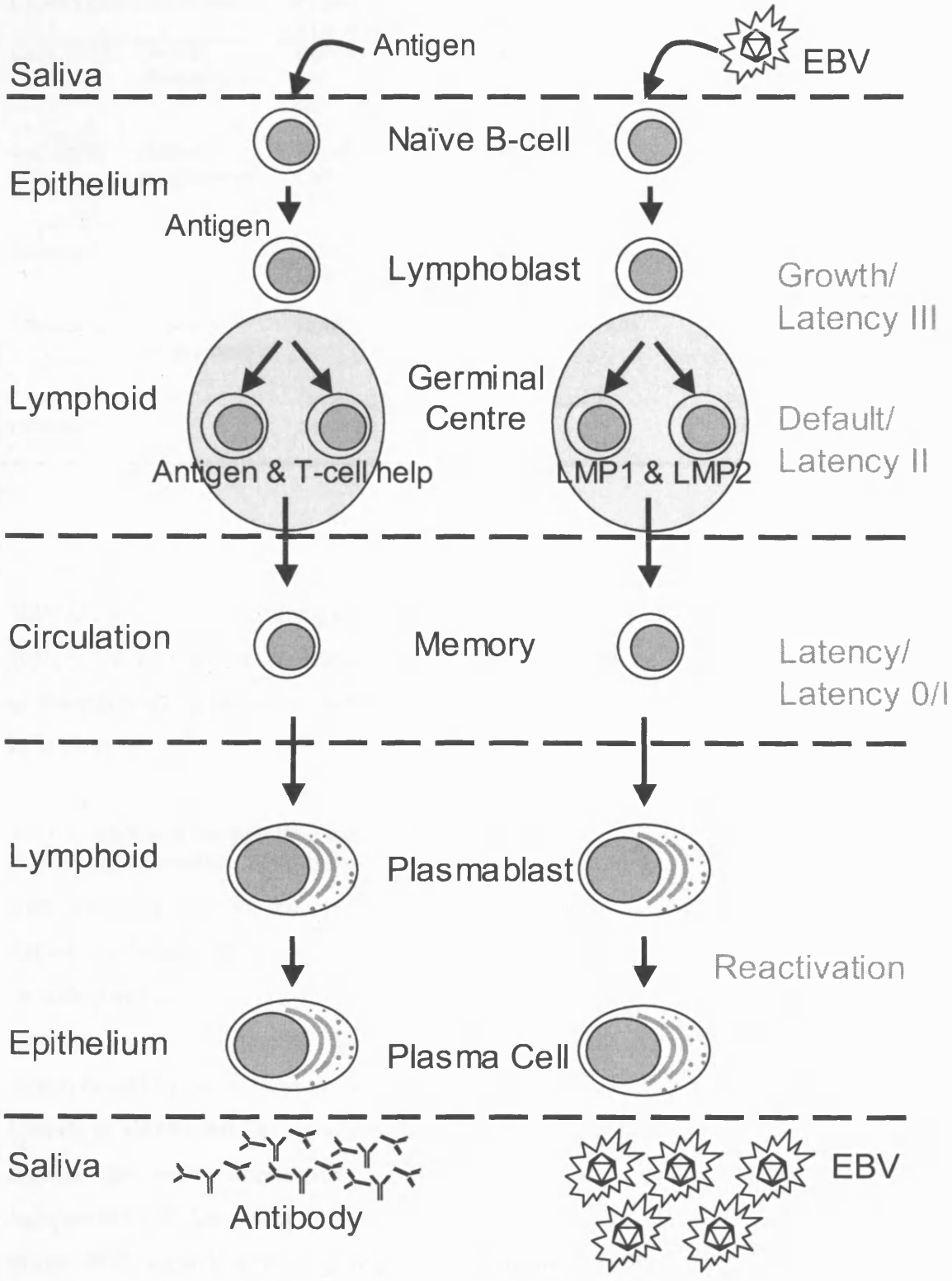


Figure 1.2.7. B-cell development and EBV (Adapted from Thorley-Lawson, 2005)
A diagrammatic representation of normal and EBV-driven B-cell differentiation. EBV gene expression programmes are highlighted in grey.

Table 1.2.7. EBV expression programmes (adapted from Thorley-Lawson, 2001).

Expression programme		Proteins expressed	Expression observed in	Function
Latency III	Growth programme	EBNA1-6 LMP1 LMP2A-B	Activated B-cell LCLs	Activates resting B-cells to form lymphoblasts
Latency II	Default programme	EBNA1 LMP1 LMP2A	GC-B-cell Classical Hodgkin's lymphoma	Episome maintenance, survival and proliferation
Latency I		EBNA-1 LMP2A transcript	Burkitt's lymphoma	Allows persistence
Latency 0	Latency Programme	None	Memory B-cells	Allows persistence
Lytic replication		Ordered lytic cascade	Plasma cells	Produces infectious virions

EBNA-2 acts as a controlling transcription factor in the growth programme/latency III. EBNA-2 is able to activate expression of viral genes (Abbot *et al.*, 1990) necessary to activate signalling pathways and 'trick' the naïve B-cell into becoming activated (Wang *et al.*, 1987).

1.2.7.2. EBV and the germinal centre reaction

Following activation, EBV-positive lymphoblasts migrate to lymphoid follicles where they can take part in the germinal centre reaction. At this point the EBV gene expression programme changes from latency III/the growth programme to latency II/the default programme. This change requires that EBNA-2 transcriptional activation is downregulated. Exactly how this is achieved is unclear, but EBNA-2 hyperphosphorylation (Yue *et al.*, 2004) has been implicated at this stage. Entering latency II allows the EBV-positive B-cell to adopt a germinal centre like phenotype. Indeed, the latent membrane protein (LMP)-2A is capable of driving antigen independent GC formation in transgenic mice (Casola *et al.*, 2004). LMP-2A is able to mimic BCR signalling through association with the tyrosine kinase lyn (Burkhardt *et al.*, 1992). The signalling induced by LMP-2A is pro-survival and LMP-2A can rescue B-cell development in immunoglobulin knockout mice (Caldwell *et al.*, 1998).

LMP-1 is able to mimic signalling through CD40 providing a signal similar to T-cell help (Zimber-Strobl *et al.*, 1996; Kilger *et al.*, 1998). LMP-1 achieves this through ligand-independent recruitment of tumour necrosis factor (TNF) receptor-associated factors (TRAFs). Interestingly, LMP-1 can also upregulate BAFF expression (He *et al.*, 2003), which can partially substitute for CD40 activation (Litinskiy *et al.*, 2002), causing ligand-independent CSR (He *et al.*, 2003).

The concerted action of LMP-2A and LMP-1 mimic the signals caused by encounter with antigen and T-cell help. To survive the germinal centre reaction, B-cells must receive these pro-survival signals to escape negative selection. Although LMP-2A and LMP-1 favour the survival of EBV-positive B-cells within the germinal centre, it is clear that there is a role for the BCR in the development of EBV-positive memory cells. EBV-positive memory cells all express a functional BCR (Souza *et al.*, 2005). This suggests that the pro-survival signals of LMP-1 and LMP-2A are not sufficient to replace BCR signalling in the generation and maintenance of memory cells.

1.2.7.3. EBV reactivation and plasma cell differentiation

Eventually, EBV-positive GC B-cells exit the germinal centre reaction. LMP-1 may be involved in this process and is capable of repressing BCL-6 expression in transgenic mice (Panagopoulos *et al.*, 2004). Exit from the germinal centre is associated with differentiation into memory cells or plasma cells, and each is considered in turn.

Memory cell differentiation of EBV-positive cells is the mechanism through which EBV achieves lifelong persistence. EBV-positive memory cells re-enter circulation and preferentially home to the tonsils and adenoids (Laichalk *et al.*, 2002). Persistence within the memory cell compartment is associated with the latency/latency 0/1 viral gene expression programmes. Minimal viral protein expression, within memory cells, allows immune evasion and, in most cases, non-pathogenic persistence.

Plasma cell differentiation is the cue for EBV reactivation (Crawford and Ando, 1986; Laichalk *et al.*, 2005). Exactly how reactivation is triggered in these cells is unclear, but reactivation may be largely abortive (Laichalk *et al.*, 2005). All EBV carriers shed EBV in their saliva (Yao *et al.*, 1989) but the number of plasma cells supporting reactivation cannot account for the magnitude of virus production. This suggests that virus production from plasma cells is subsequently amplified in the oral mucosa

(Laichalk *et al.*, 2005). Persistence within the memory pool coupled to reactivation in plasma cells allows EBV to maintain lifelong infection, yet continually produce infectious virus through antigen-independent memory cell activation (Bernasconi *et al.*, 2002). The plasma cell also represents an ideal environment for herpesvirion production and the ability of plasma cells to synthesise, fold and secrete large quantities of protein may inadvertently have adapted plasma cells for virus production.

1.2.8. The aims of this thesis

The difficulty of isolating KSHV-positive B-cells from healthy individuals coupled to the inability of KSHV to form LCLs *in vitro* (Kliche *et al.*, 1998) has largely restricted the understanding of KSHV latency in B-cells to PEL-derived cell lines. We hypothesize that, similarly to EBV, KSHV has a lifecycle which is intertwined with B-cell differentiation. The observation that KSHV is latent in PEL cells, which resemble plasmablasts (Jenner *et al.*, 2003), but is lytic in a minority of cells within MCD lesions (Parravicini *et al.*, 2000; Katano *et al.*, 2000) led us to consider that, similarly to EBV, plasma cell differentiation is a cue for KSHV reactivation.

The low susceptibility of B-cell lines to infection with KSHV *in vitro* is an enigmatic feature of KSHV biology. We hypothesize that *in vitro* susceptibility is related to the B-cell developmental stage and activation state of the target B-cell. It is possible that the recalcitrant nature of some B-cell lines, to KSHV infection, may be a consequence of those cell lines representing a stage or subset of B-cells not targeted by KSHV *in vivo*.

Finally, the difficulty in isolating and generating KSHV positive B-cells has led us to explore alternative approaches for investigating KSHV-B-cell relationships. This led us to consider the utility of RNA interference at specifically attenuating viral or host cell gene expression within PEL-derived cell lines and RNA interference is considered in section 1.3.

1.3. RNA interference (RNAi)

1.3.1. The history of RNAi

The term RNA interference (RNAi) was first used to describe an interference with gene expression caused by introducing sense or antisense RNA into *C. elegans* embryos (Fire *et al.*, 1991 and Guo and Kemphues, 1995). The key breakthrough in understanding this phenomenon was the realisation that double stranded RNA (dsRNA) was the most effective mediator of RNAi. Introducing dsRNA into *C. elegans* resulted in mRNA degradation with the sequence of the dsRNA specifying which transcripts were degraded (Fire *et al.*, 1998). It rapidly became clear that RNAi pathways were conserved across a wide variety of eukaryotes and the same phenomenon, termed "cosuppression" or "post transcriptional gene silencing" was already the subject of intensive research in plants (Napoli *et al.*, 1990, van der Krol *et al.*, 1990).

A significant barrier existed before this technology could be exploited experimentally in mammalian cells. Double stranded RNA was well known to shut-off translation in mammalian cells (Ehrenfeld and Hunt, 1971). This phenomenon is caused in part by protein kinase R (PKR), which when activated through binding dsRNA greater than 30 base pairs (bp) in length (Manche *et al.*, 1992) homodimerises and undergoes autophosphorylation (Zhang *et al.*, 2001). This creates an active PKR homodimeric complex capable of phosphorylating eukaryotic initiation factor 2 (eIF2) preventing translation initiation (Farrell *et al.*, 1977). It was unclear, however, if long dsRNA-mediated RNAi could be used specifically without activating innate pathways sensitive to dsRNA (described in section 1.3.5.2).

Conservation of the RNAi machinery in mammalian cells was demonstrated using long RNA hairpins, greater than 500 bp in length, in mouse preimplantation embryos to achieve silencing (Svoboda *et al.*, 2001). However, it was not until further characterisation of the mechanism of RNAi that the importance of mammalian RNAi became more widely appreciated and experimentally applicable. In 2000 it was realised that long dsRNAs were processed to 20-25 bp small/short interfering RNAs (siRNAs) and that these were incorporated into a RNAi-effector complex capable of degrading target mRNAs (Zamore *et al.*, 2000; Hammond *et al.*, 2000). By transfecting chemically synthesized 21 bp siRNAs, thought to be too short to activate dsRNA

sensitive pathways, Tuschl and colleagues were able to suppress endogenous and exogenous gene expression in human cell lines (Elbashir *et al.*, 2001a). The discovery of RNAi as a novel control mechanism of gene expression and the utility of this mechanism in experimental systems has resulted in an explosion of interest in RNAi and its rapid adoption as a standard technique in molecular biology (figure 1.3.1.1).

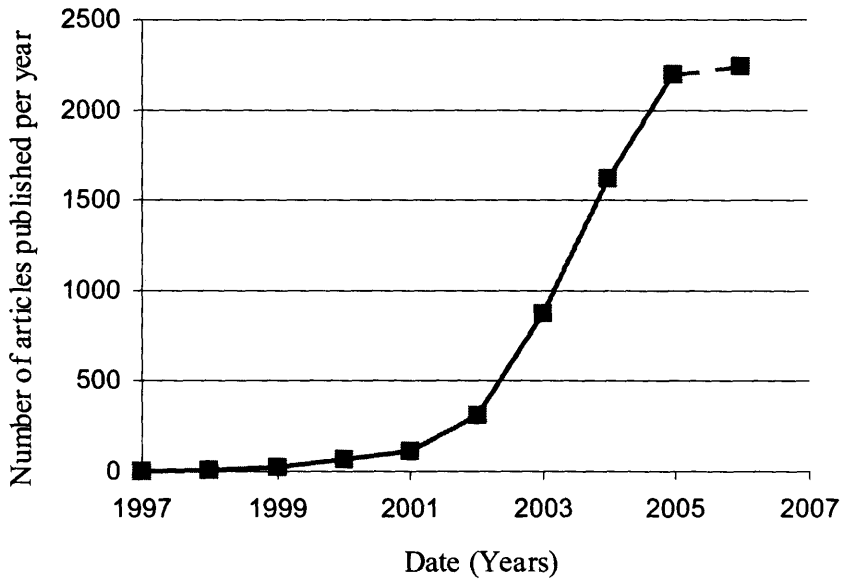


Figure 1.3.1.1. Rapid increase in publications related to RNAi.
Number of publications returned using the integrated, text-based search and retrieval system Entrez returned from the PubMed database using the query "RNAi". A 2006 estimate is included based upon publications prior to April 1st, 2006.

Since its discovery, the RNAi machinery and pathways have become increasingly well characterised (reviewed in Meister and Tuschl, 2004; Zamore and Haley 2005; Filipowicz, 2005) and a wide variety of naturally expressed microRNAs have been identified and their activity characterised (reviewed in Ambros, 2004; Sontheimer and Carthew 2005; Pasquinelli *et al.*, 2005). Features affecting siRNA potency have become increasingly well defined (reviewed in Silva *et al.*, 2003) and RNAi as a therapy has entered clinical trials. A summary of some of the 'landmarks' in RNAi is illustrated in figure 1.3.2.1.

1.3.2. The mechanisms of RNAi

Depending on their origin, small RNAs can be divided into three categories short/small interfering RNAs (siRNAs), microRNAs (miRNAs) and repeat-associated small interfering RNAs (rasiRNAs). All 3 classes tend to operate in different pathways, using complexes which may be identical but effect different methods of gene silencing (Figure 1.3.2.2).

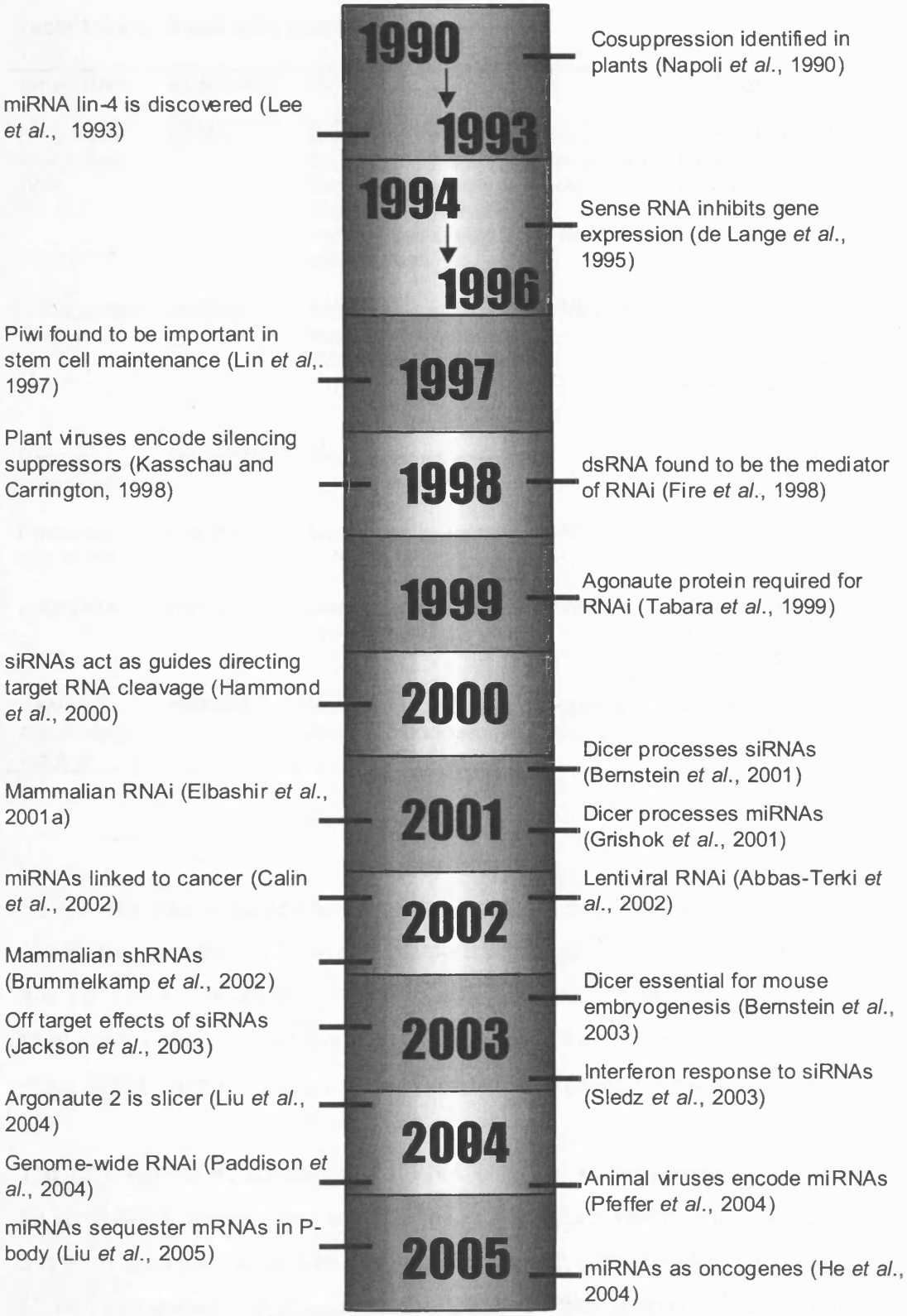


Figure 1.3.2.1. A timeline of significant discoveries related to RNAi.
Aadapted from Zamore and Haley, 2005 and Godfrey *et al.*, 2003.

dicers (Lee *et al.*, 2004) and dicers from different species can produce siRNAs of different lengths (Bernstein *et al.*, 2001; Ketting *et al.*, 2001) and have differing ATP requirements. Human cells express a single dicer, whose enzymatic activity is ATP independent, which starts 'dicing' at the ends of long dsRNA (Zhang *et al.*, 2002).

As well as a requirement in generating siRNAs, dicer seems to have a pivotal role in incorporating siRNAs into the RISC. RNAi-mediated knockdown of dicer in HeLa cells inhibits siRNA mediated silencing of the reporter gene luciferase (Doi *et al.*, 2003) and introduction of siRNAs into *dcr-2* null embryos fails to achieve silencing at levels similar to wild-type embryos (Lee *et al.*, 2004). This suggests that dicer's involvement in RNAi stretches beyond generating siRNAs. Indeed, short 27 bp dsRNA dicer substrates seem more potent effectors of silencing than their 21 nt siRNA counterparts (Kim *et al.*, 2005)

A breakthrough in siRNA design was facilitated by determining dicer's role in silencing downstream of generating siRNAs. It is generally accepted that dicers are capable of interacting with Argonaute (Ago) proteins a key component of the RISC (Hammond *et al.*, 2001; Tabara *et al.*, 2002; Tahbaz *et al.*, 2004). Simultaneous biochemical (Schwarz *et al.*, 2003) and bioinformatics (Khvorova *et al.*, 2003) approaches identified an asymmetry in the siRNA strand incorporated into the RISC. This asymmetry involves the preferential loading of the siRNA strand whose 5' end resides at the RNA duplex terminus with the lowest free energy (reviewed in Silva et al., 2003). *D. melanogaster* dicer-2 binds siRNAs as a heterodimer with the protein R2D2 (Liu *et al.*, 2003) forming a ternary complex termed the RISC-loading complex (RLC). Photocrosslinking of siRNAs within this complex identified preferential binding of dicer to the less stable siRNA terminus and preferential binding of R2D2 to the more stable siRNA terminus (Tomari *et al.*, 2004) the structural determinants of this selectivity have yet to be determined. The asymmetric formation of the RLC probably represents the step at which the siRNA guide strand is selected (reviewed in Filipowicz, 2005). The asymmetry rule seems applicable to all organisms investigated and R2D2 homologues and the human immunodeficiency virus transactivating response RNA-binding protein (TRBP) seems functionally analogous to R2D2 in humans (Chendrimada *et al.*, 2005). Overexpressed TRBP has been shown to inhibit PKR activity (Park *et al.*, 1994) but any functional relevance of this interaction in RNAi has yet to be identified.

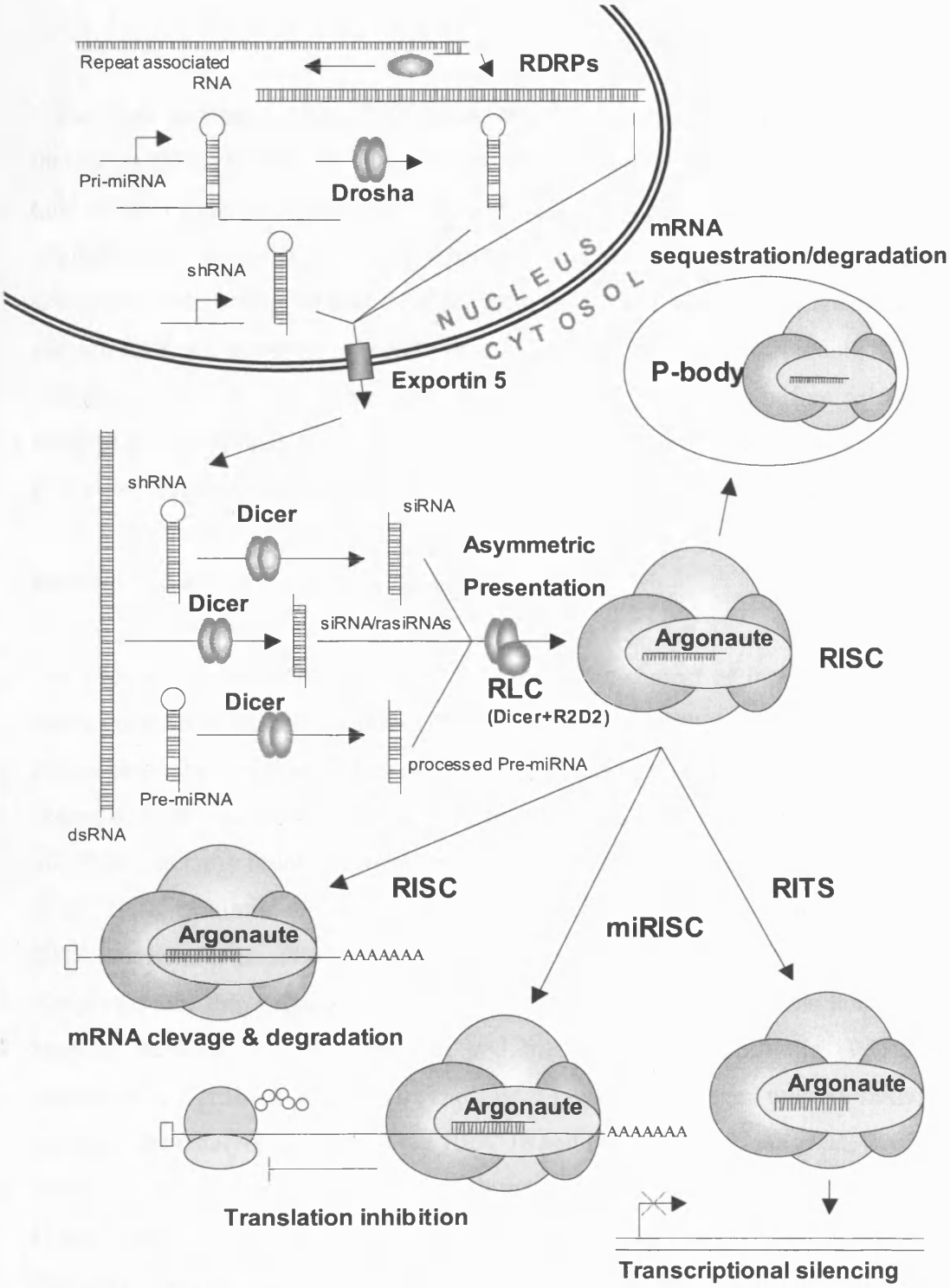


Figure 1.3.2.2. The mechanisms of RNAi
A diagrammatic representation of RNAi is shown. Repeat associated RNA, pri-miRNAs and shRNAs are transcribed in the nucleus. Complementary repeat associated RNA is synthesised by RDRPs. Pri-miRNAs are Drosha-processed, generating pre-miRNAs. Pre-miRNAs, shRNAs and ssRNA are exported from the nucleus where they become dicer substrates resulting in the generation and asymmetric presentation of siRNAs, rasiRNAs and miRNAs to the RISC. The 4 possible modes of RISC action are indicated; Cleavage of mRNA by the RISC, inhibition of translation by the miRISC, mRNA sequestration in the P-body and transcriptional silencing by the RITS. pri-miRNA, primary miRNA; shRNA, short hairpin RNA; RDRPs, RNA dependent RNA polymerases; ssRNA, single stranded RNA; siRNA, small interfering RNA; rasiRNAs, repeat associated small interfering RNA; miRNA, microRNA; RISC, RNA-induced silencing complex; miRISC, miRNA induced silencing complex; P-body, processing body; RITS, RNA induced transcriptional silencing complex.

If the RLC determines the siRNA strand which is loaded as a guide, the observations that dicer directs binding to long dsRNA at the RNA termini (Zhang et al., 2002) but bind siRNAs in an orientation determined by RNA duplex stability (Tomari *et al.*, 2004) are difficult to reconcile. These apparently conflicting observations could mean that the asymmetry rule should be broken approximately 50% of the time when dicer processes long dsRNA and is unable to select the less stable duplex terminus, binding the exposed terminus. Alternatively, dicer could release processed siRNAs before selectively re-binding them for presentation to the RISC. A mechanistic consensus has yet to arise but it is clear that dicer-processed miRNAs are incorporated asymmetrically into the RISC *in vivo* (Khvorova *et al.*, 2003) supporting the notion that dicer processed RNAs are presented asymmetrically by the RLC.

The protein composition of the RISC has been the subject of intensive research, and many complexes have been isolated, their catalytic activity analysed and their protein components characterised. Most of this analysis has been done using Drosophila S2 cell extracts. RISC complexes can be isolated associated with polysomes at 80S (Pham *et al.*, 2004), as large holoRISC complexes at ~500 kDa (Nykanen *et al.*, 2001; Hammond *et al.*, 2001) or minimal complexes isolated under high salt conditions (Martinez *et al.*, 2002; Martinez *et al.*, 2004). The only protein components present in all isolated RISC complexes are the Argonaute (Ago) family members. Ago proteins contain 2 RNA binding domains, the piwi domain and the piwi/Argonaute/zwille (PAZ) domain (reviewed in Cerutti *et al.*, 2000). The Ago 2 protein complexed with an ssRNA guide mediates the catalytic activity of the RISC (Rand *et al.*, 2004; Rivas *et al.*, 2005). The catalytic activity resides in the piwi domain which is structurally homologous to RNase H and is dependent on the triad motif DDH which is absent in other Argonaute family members (Liu *et al* 2004; Rivas *et al.*, 2005). Although the mechanism of RNA transfer from the RLC to the RISC remains obscure, structural studies indicate that the 5' end of the siRNA is anchored in the piwi domain (Parker *et al.*, 2004) and the 3' end in the PAZ domain (Lingel *et al.*, 2004). The Ago-2 guide RNA complex is able to scan mRNA, and in the presence of sufficient complementary base pairing, catalyse endonucleolytic cleavage of the mRNA between bases 10-11 of the RNA guide (Elbashir *et al.*, 2001b). Human Ago 2 complexed with RNA is competent to undergo a single round of cleavage in the absence of ATP (Rivas *et al.*, 2005), but in the presence

of additional protein factors and ATP, Ago-2 forms the catalytic heart of a multiple-turnover enzyme complex capable of directing multiple rounds of mRNA cleavage (Hutvagner and Zamore 2002). Once cleaved the mRNA is rapidly degraded by exonucleases (Orban and Izaurralde, 2005), reducing mRNA abundance of a targeted transcript resulting in reduced protein expression from a targeted mRNA.

1.3.2.2. MicroRNAs and repression of translation

The *C. elegans* miRNA *lin-4* was the first microRNA (miRNA) to be identified (Lee *et al.*, 1993). However it was not until a role for dicer in miRNA processing was identified that miRNAs were linked to other RNAi phenomena (Grishok *et al.*, 2001). It is important when considering miRNAs to remember that the term miRNA refers to its origin and not the mode of action (Table 1.3.2.1.). Indeed plant miRNAs tend to mediate RNAi in a canonical RISC complex, cleaving target mRNAs (Llave *et al.*, 2002; Rhoades *et al.*, 2002) and transfected siRNAs can act in a miRNA-induced silencing complex (Doench *et al.*, 2003). The miRISC is therefore defined by its action in repression of target mRNA translation, not by the origin of its small RNA component.

The database miRBase (http://microrna.sanger.ac.uk), formerly the microRNA registry (Griffiths-Jones, 2004) catalogues over 3000 miRNAs. MicroRNAs have been identified in plants, metazoans and herpesviruses. The control of gene expression by miRNAs seems to be an essential process in most organisms and miRNA regulation has been documented in many cellular processes (reviewed in Ambros, 2004). MicroRNAs are important in stem-cell division (Hatfield *et al.*, 2005), haematopoiesis (Chen *et al.*, 2004), oncogenesis (He *et al.*, 2005), cancer-classification (Lu *et al.*, 2005c) and regulate large numbers of mRNAs helping to define the global gene expression profile (Lim *et al.*, 2005).

MicroRNAs are ~22 nt RNAs derived from long, largely unstructured, usually noncoding RNA genes called primary miRNAs (pri-miRNAs). These transcripts are capped, polyadenylated and transcribed by RNA polymerase II (Cai *et al.*, 2004). Hairpin structures ~70 nt in length present in the pri-miRNA are excised from pri-mRNAs by a Microprocessor complex consisting of the enzyme Drosha and the DiGeorge syndrome critical region gene 8 (DGCR8) protein (Gregory *et al.*, 2004). These hairpins, termed pre-miRNAs, are exported from the nucleus by exportin 5 (Yi *et*

al., 2003) where they are processed by dicer enzymes (Grishok *et al.*, 2001). It is clear that similar asymmetry rules to siRNAs apply to miRNAs (Khvorova *et al.*, 2003) implying similar asymmetric dicer-TRBP binding of miRNAs and presentation to the miRISC.

Characterisation of protein components of the miRISC has failed to identify any specific protein components conferring inhibition of translation to the miRISC. Many if not all the protein components are shared between the RISC and miRISC complexes and it is possible that the only difference between a RISC mediating mRNA cleavage and a miRISC mediating inhibition of translation lies in the ability of a guide RNA to form a helix with the target mRNA (reviewed in Filipowicz, 2005). Indeed, inhibition of translation might represent the default pathway of RNAi as tethering of Argonaute proteins to mRNAs results in inhibition of translation (Pillai *et al.*, 2004). The mechanism by which the miRISC prevents target mRNA translation has yet to be determined. However, it is known that inhibition occurs at or just after translation initiation (Olsen and Ambrose 1999; Pillai *et al.*, 2005a). In addition to translation inhibition, the miRISC is capable of sequestering target mRNAs by moving them to cytoplasmic P-bodies (Liu *et al.*, 2005; Sen and Blau, 2005). In P-bodies Argonaute proteins associate with enzymes involved in mRNA degradation (Liu *et al.*, 2005; Sen and Blau, 2005) accounting for the accelerated target mRNA turnover observed in the presence of miRNAs (Lim *et al.*, 2005). The combination of inhibition of target mRNA translation and accelerated target mRNA degradation is responsible for the reduction of target protein expression caused by RNAi through the miRISC pathway.

1.3.2.3. The RNA induced transcriptional silencing (RITS) complex

RNA induced transcriptional silencing involves the suppression of mRNA transcription through DNA methylation and histone modification (reviewed in Bernstein and Allis, 2005). This process is most characterised in lower eukaryotes where the RITS complex has been purified (Verdel *et al.*, 2004) and RITS complexes can be found bound to regions of heterochromatin such as centromeres and telomeres (Volpe *et al.*, 2002). RITS complexes usually contain repeat associated siRNAs (rasiRNAs) generated from long mRNAs transcribed from regions of repetitive sequence usually amplified by RNA-dependent RNA polymerases to generate dsRNA (Sugiyama *et al.*, 2005) and processed by dicers. The absence of RNA-dependent RNA polymerases and lower levels of repeat associated RNA have hampered characterisation of this pathway in

mammalian cells. However it is clear that siRNAs can direct DNA methylation in mammalian cells (Kawasaki and Taira, 2004; Morris *et al.*, 2004) and the RNAi machinery may be involved in regulating mammalian centromeres (Fukagowa *et al.*, 2004) But the importance and generality of RITS remains unclear.

1.3.3. The technology of RNAi

After demonstration of transfected siRNA efficacy in cultured mammalian cells (Elbashir *et al.*, 2001a), siRNA transfection was rapidly adopted as an experimental tool in mammalian cell culture (Garrus *et al.*, 2001, Cortez *et al.*, 2001; Harborth *et al.*, 2001). The transfection of siRNAs usually involves chemically synthesised RNAs protected at their 3' termini with deoxythymidine dinucleotides. Lipid-based transfection reagents are then used to deliver these artificial siRNAs to the cytoplasm. Although transfected siRNAs are the most common experimental mediator of RNAi, they were not used in experiments detailed in this thesis and are not discussed further.

The realisation that hairpin pre-miRNAs expressed *in vivo* are processed by dicer (Grishok *et al.*, 2001) led to the development of DNA vectors capable of expressing short hairpin RNAs (shRNAs), which analogous to pre-miRNAs could be processed by dicer generating siRNAs. Two initial reports detailed use of the human H1-promoter (Brummelkamp *et al.*, 2002) and the murine U6-promoter (Sui *et al.*, 2002) to achieve gene silencing. Both promoters are RNA polymerase III (pol III) type-3 promoters requiring no intragenic enhancers, a common feature of other RNA pol III promoters (reviewed in Willis, 1993). Although many different promoters have been used to deliver shRNAs, The H1 and U6-promoters remain the most popular promoters and no consensus has emerged regarding the most effective shRNA design and delivery system.

Chapter 3 of this thesis details the generation of an shRNA expression cassette. The anatomy of an shRNA is detailed in figure 1.3.3.1. For an shRNA to be functional in gene silencing it must contain sufficient helical dsRNA to act as a substrate for dicer. This can be achieved with duplexes as short as 19 bp (Brummelkamp *et al.*, 2002) although longer stems seem to be more favourable substrates for dicer, resulting in more potent gene silencing (Siolas *et al.*, 2005). The primary function of the loop region is to structurally permit helical interaction of the two strands of the duplex stem. Dicer processing of shRNAs is a cytoplasmic process (Lee *et al.*, 2002c) and shRNAs must be exported from the nucleus, probably using exportin 5 (Yi *et al.*, 2003) to be processed

into siRNAs. Making shRNA loops similar to miRNA loops has been reported to increase the efficiency of silencing, possibly due to enhanced shRNA export (Miyagishi *et al.*, 2004). The molecular basis of any increased silencing, in shRNAs engineered to contain miRNA loops, is unclear because exportin 5 and dicer seem unable to recognise shRNA loops (Zeng and Cullen, 2004; Vermeulen *et al.*, 2005). Many shRNAs are designed to contain GU basepairs within the duplex stem. The presence of GU basepairs reduces DNA secondary structures easing shRNA cloning and sequencing. Introducing GU basepairs is usually achieved by mutating only the sense strand of an shRNA, relative to the target mRNA. This retains Watson-Crick basepairing between

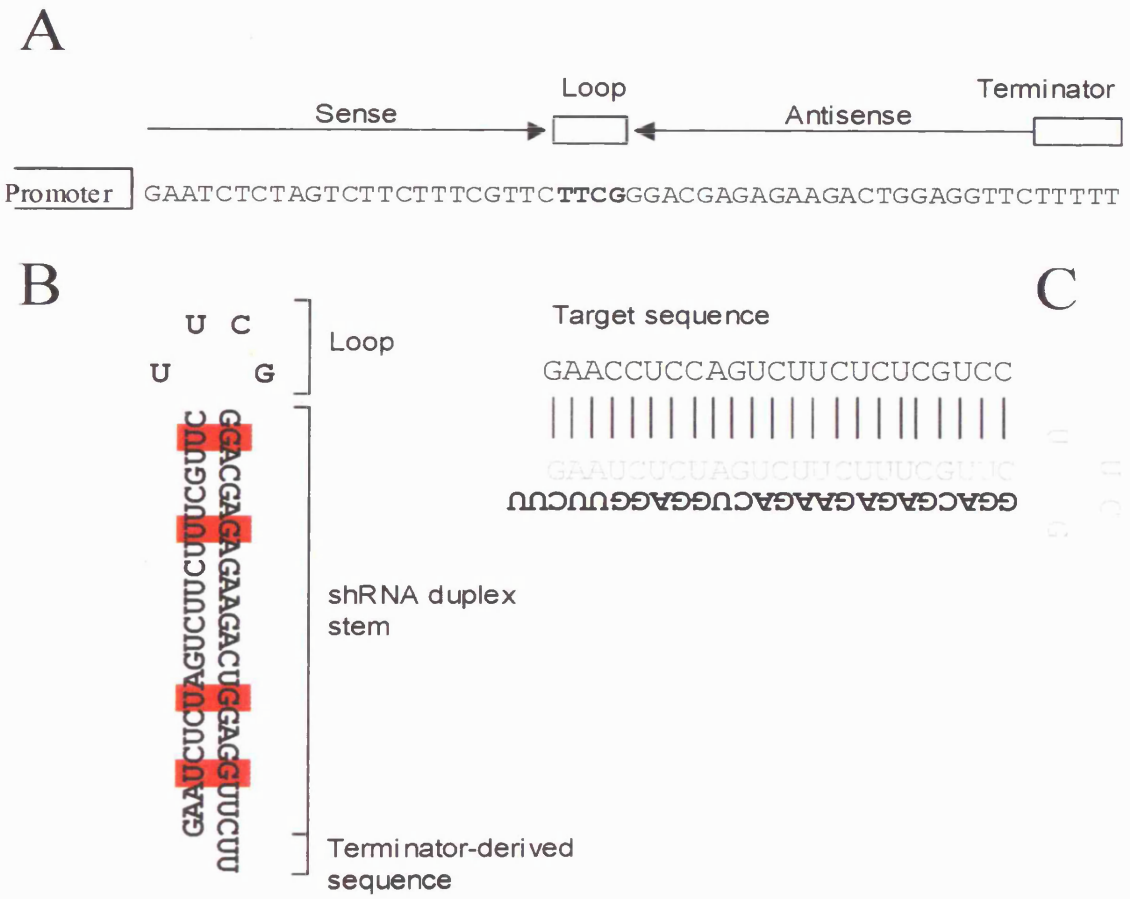


Figure 1.3.3.1. The anatomy of an shRNA.
A. A diagrammatic representation of an shRNA expression cassette. The DNA template is displayed 5'-3' and loop and transcription terminator sequences are indicated. The sense and antisense strands of the stem duplex are indicated with arrows. B. A diagrammatic representation of the predicted shRNA expressed from the template in A. The loop, stem and terminator-derived RNA are indicated. GU basepairs are highlighted in red. C. A diagram indicating that the antisense strand of the shRNA is capable of perfect Watson-Crick basepairing with the target mRNA. Sequences other that the target mRNA sequence and the antisense shRNA strand are displayed in grey type.

the guide RNA and the target mRNA. Transcription termination of shRNAs is achieved by a run of 5 or more consecutive thymidines, as is the case for the H1 (Baer *et al.*, 1990) and U6 (Kunkel and Pederson, 1988) small RNA genes.

The shRNA expression cassette detailed in this thesis utilises the human U6-promoter. The expressed U6 small nuclear RNA exists in a small nuclear ribonucleoprotein complex which is involved in spliceosome assembly and catalysis of pre-mRNA splicing (reviewed in Butcher and Brow, 2005). The U6-promoter is suitable for shRNA mediated RNAi because the promoter is capable of expressing small RNAs to a very high level, approximately $4x10^5$ per cell (Weinberg and Penman, 1968), and requires no intragenic transcription enhancers. Exogenously expressed small RNA transcripts, initiating with guanine, driven by the U6-promoter are easily detectable (Good *et al.*, 1997). Because the sequences required for capping of U6 snRNAs are intragenic (Singh *et al.*, 1990; Good *et al.*, 1997) the U6-promoter is capable of expressing large amounts of uncapped shRNAs, an RNA species similar to pre-miRNAs, which can mediate potent RNAi (Sui *et al.*, 2002).

With regard to siRNA and shRNA design, it is not yet possible to predict the potency of a siRNA from its sequence. However, it is clear that the stability of duplex RNA ends determines which strand is preferentially incorporated into the RISC and this constraint should be considered when designing siRNAs (Reynolds *et al.*, 2004; Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). Many programmes are currently available to assist in siRNA/shRNA design. Most of these programmes are based upon energetic constraints related to the asymmetry rule as well as constraints related to shRNA cloning and processing (Vermeulen *et al.*, 2005; Siolas *et al.*, 2005). A consensus has yet to emerge regarding which method designs the most potent siRNAs.

1.3.4. Lentiviral vectors

This thesis describes the use of lentiviral vectors both to deliver shRNA expression cassettes to a variety of cells (chapter 3) and to deliver the transcription factor XBP-1 to PEL cell lines (chapter 5). Lentiviral vectors are one of the most flexible gene delivery systems available, capable of transducing a wide variety of cells and delivering

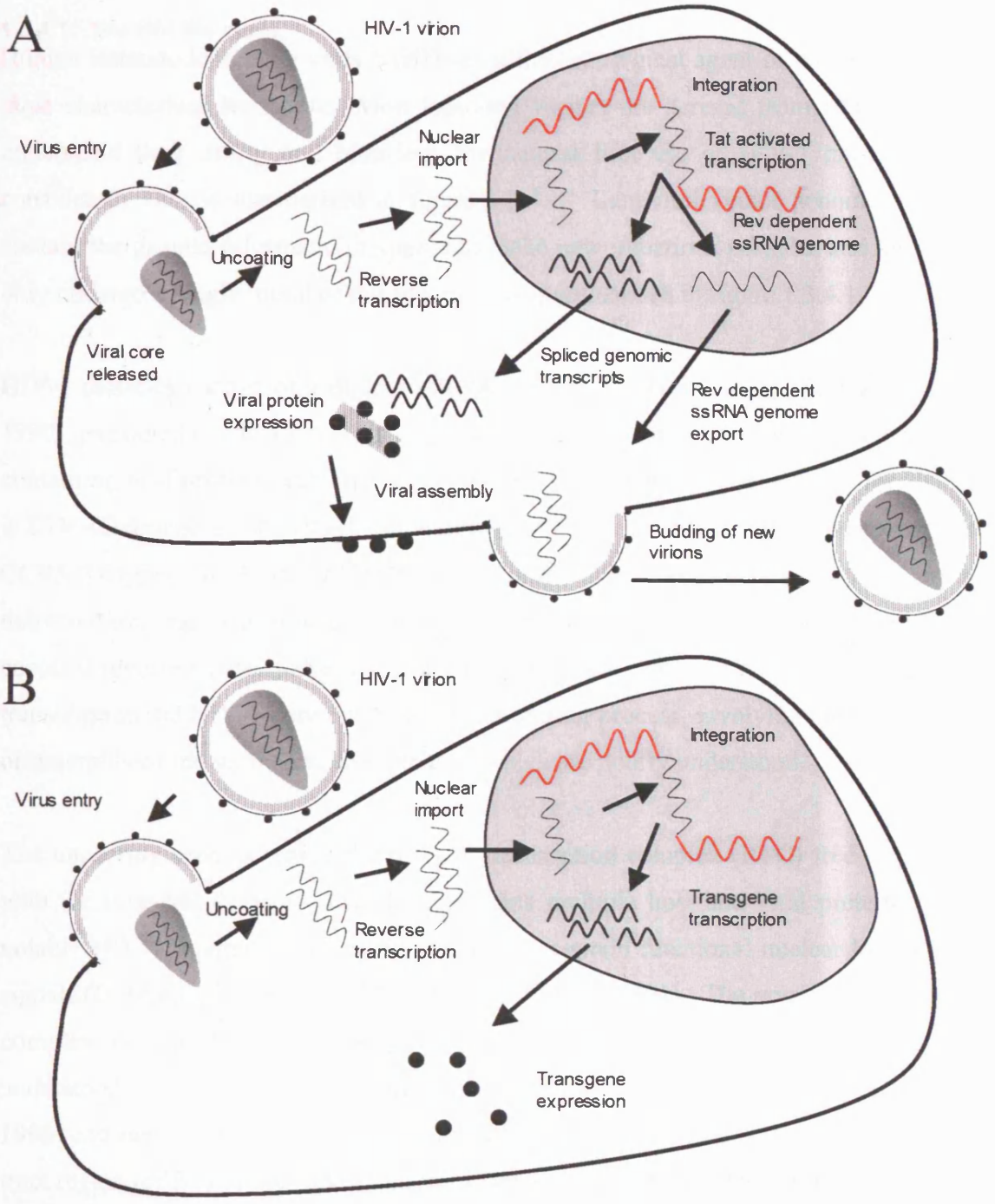


Figure 1.3.4.1. The replication cycle of HIV-1 and single round transduction by lentiviral vectors.
A. A diagrammatic representation of the HIV-1 replication cycle. B. Diagrammatic representation of transduction with a lentiviral vector.

sustained heritable expression of transgenes (reviewed in Buchschacher and Wong-Staal, 2000; Quinonez and Sutton, 2002; Wiznerowicz and Trono 2005).

1.3.4.1. The HIV life cycle

Human immunodeficiency virus 1 (HIV-1) is the aetiological agent of AIDS and is the most characterised lentivirus. Most lentiviral vectors are derived from HIV-1 and to understand their utility and biosafety, the natural lifecycle of HIV-1 must first be considered. This is summarised in figure 1.3.4.1. Lentiviral vector genomes do not contain the genetic information required to make new infectious particles and therefore only undergo a single round of transduction also summarised in figure 1.3.4.1.

HIV-1 particles consist of a diploid ssRNA genome 9.3 kb in length (Schwartz *et al.*, 1990), packaged inside a retroviral core which in turn is surrounded by a lipid envelope containing host proteins and viral glycoproteins. The main cellular receptor for HIV-1 is CD4 (Dalgleish *et al.*, 1984). In addition, HIV-1 entry also requires the coreceptor CCR5 (Dragic *et al.*, 1996) or CXCR4 (Feng *et al.*, 1996). After fusion, the viral core is delivered into the cytosol where the genomic RNA is reverse transcribed by the HIV-1 encoded reverse transcriptase which is packaged within the viral core. During reverse transcription the HIV-1 core undergoes an uncoating process, involving the progressive disassembly of the viral core, the details of which are poorly understood.

The uncoating process leaves the reverse transcription complex (RTC) free to interact with the host-cell cytosol. The RTC contains multiple host and viral proteins, most notably HIV-1 integrase and matrix which may contain functional nuclear localisation signals (Dubrovsky *et al.*, 1995; Bouyac-Bertoia et al., 2001). The reverse transcription complex is capable of traversing the nuclear envelope. This process is poorly understood, but is active (Bukrinsky *et al.*, 1992) involves nuclear pores (Popov *et al.*, 1998) and may even require structural DNA-RNA hybrids within the central polypurine tract region (cPPT) of the HIV-1 genome (Zennou *et al.*, 2000). The ability of HIV-1 to cross the nuclear pore allows HIV-1 to infect quiescent nondividing cells, such as macrophages (Weinberg *et al.*, 1991).

Once inside the nucleus, integration of HIV-1 double stranded DNA into the host genome ensues. Integration is catalysed by HIV-1 integrase and is not sequence specific. However, HIV-1 integration is more frequent in transcriptionally active

regions of the chromosome (Schroder *et al.*, 2002). Once integrated, the virus is referred to as a provirus.

HIV-1 genes transcribed from the integrated provirus, are expressed from a single promoter region within the 5' long terminal repeat (LTR). The HIV-1 LTR can be divided into 3 regions; the 450 bp unique 3' (U3) region, a 100 bp repeat (R) region and a 180 bp unique 5' (U5) region. Transcription from the LTR results in a single 9.3 kb transcript which can be spliced into over 30 mRNA species (Schwartz *et al.*, 1990). Most elements required to drive transcription reside within the U3 region of the LTR, and transcription initiates within the R region of the 5' LTR. Transcription from the LTR is activated 100 to 500-fold by the HIV-1 Tat protein (Jones and Peterlin, 1994). Tat does not bind the proviral DNA LTR directly. Instead Tat activates transcription via an interaction with a stem-loop structure at the 5' end of the nascent RNA transcript termed the transactivating response (TAR) RNA. TAR-RNA is an approximately 60 bp stem loop similar in structure to pre-miRNAs and is also bound by TRBP (Gatignol *et al.*, 1991).

In the absence of viral proteins, the nascent transcript is extensively spliced. To produce the full-length genomic transcript, capable of nuclear export and packaging into new lentiviral particles, the HIV-1 regulator of expression of virion (Rev) protein is required. Rev binds to a 351 nucleotide (nt) region of secondary structured RNA within the envelope coding region (*env*) referred to as the Rev response element (RRE) (Daly *et al.*, 1989) and is able to mediate nuclear export of unspliced and partially spliced RNAs (Fischer *et al.*, 1994). The full-length ssRNA genome is then complexed with HIV-1 structural *gag*-derived proteins and buds through cellular membranes (reviewed in Kaplan *et al.*, 2002), forming an immature viral core. Following budding, these immature particles undergo HIV-1 protease mediated maturation to generate enveloped infectious virions.

Although HIV-1 derived lentiviral vectors had existed for some time (Page *et al.*, 1990), the utility of HIV-1 as a gene-delivery vector was only demonstrated in 1996 when Didier Trono and colleagues demonstrated the ability of lentiviral vectors to transduce nondividing neurons (Naldini *et al.*, 1996). These first generation lentiviral vectors were generated by transient transfection of HEK 293-T cells with 3 plasmids: a plasmid encoding the minimal ssRNA genome, a plasmid encoding all HIV-1 proteins excluding

the envelope and a plasmid encoding envelope glycoproteins. This 3-plasmid transfection system provides the first facet of lentiviral vector biosafety. By separating the nucleic acids encoding the protein constituents of a virion from the viral vector-

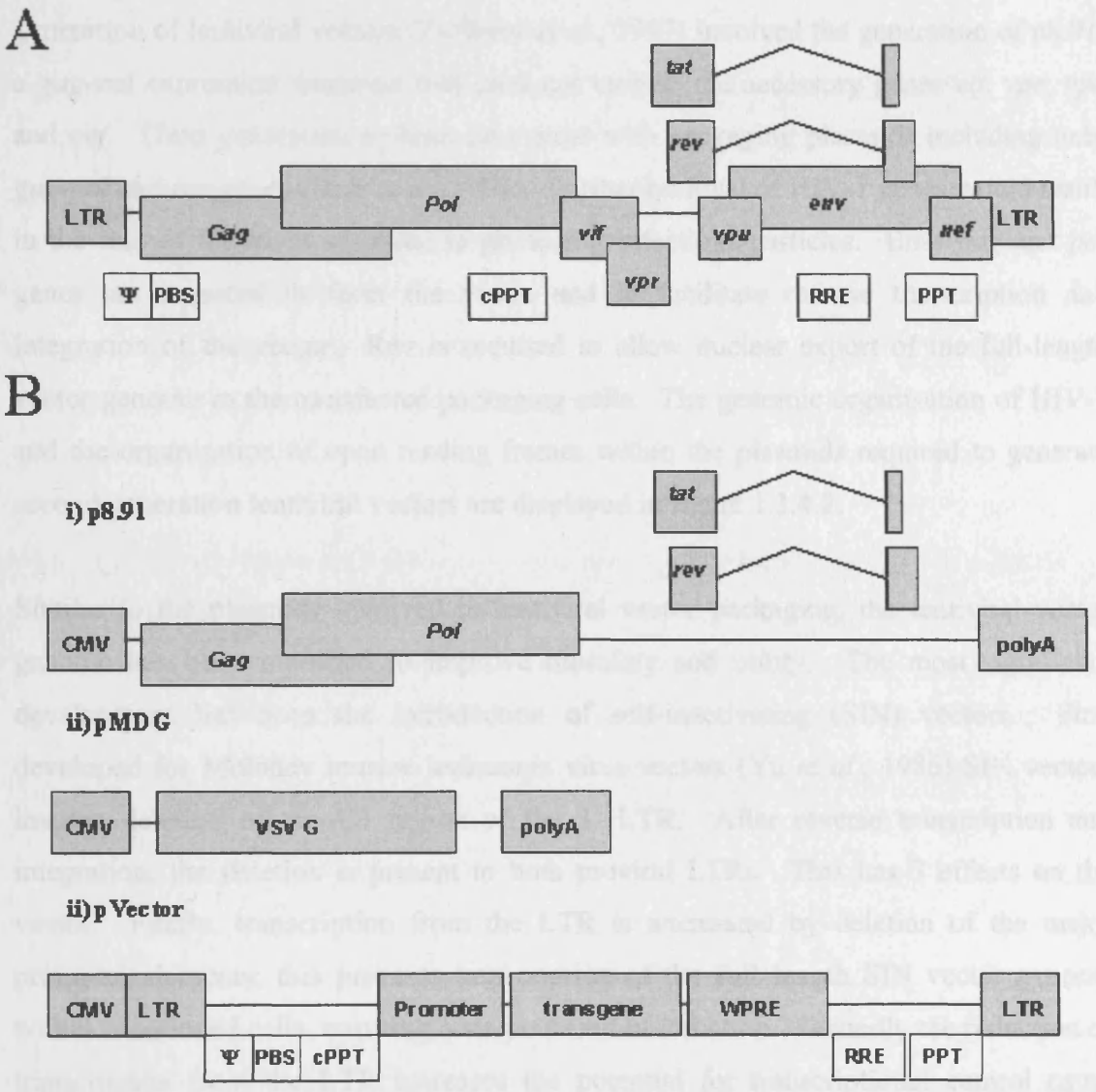


Figure 1.3.4.2. The genomic organisation of HIV-1 and the genetic organisation of expression plasmids used to make a typical second generation lentiviral vector.
A. A diagrammatic representation of HIV-1 genomic organisation. B. A diagrammatic representation of the genetic organisation of expression vectors required to generate a typical 2nd generation lentiviral vector. Shaded boxes represent genes or promoters and non-shaded boxes represent *cis*- acting elements.

genome, multiple recombination events would be required between the plasmids to generate a replication competent virus. The HIV-1 envelope severely limits the range of cells that could be transduced by HIV-1 vectors. This has lead to the use of plasmids expressing vesicular stomatitis G (VSV-G) envelope protein in the place of plasmids expressing the HIV-1 envelope protein (Naldini *et al.*, 1996). This generates pantropic, pseudotyped lentiviral vectors. The potential use of lentiviral vectors in gene therapy spurred rapid improvements in the biosafety of lentiviral vectors. The second generation of lentiviral vectors (Zufferey *et al.*, 1997) involved the generation of p8.91, a *gag-pol* expression construct that does not include the accessory genes *vif*, *vpr*, *vpu* and *nef*. Third generation systems now exist with packaging plasmids including only *gag-pol* and *rev* genes (Dull *et al.*, 1998). Further removal of HIV-1 genes would result in the loss of functions essential in producing infectious particles. Both *gag* and *pol* genes are required to form the virion and to facilitate reverse transcription and integration of the vector. Rev is required to allow nuclear export of the full-length vector genome in the transfected packaging cells. The genomic organisation of HIV-1 and the organisation of open reading frames within the plasmids required to generate second generation lentiviral vectors are displayed in figure 1.3.4.2.

Similar to the plasmids involved in lentiviral vector packaging, the lentiviral vector genome has been modified to improve biosafety and utility. The most significant development has been the introduction of self-inactivating (SIN) vectors. First developed for Moloney murine leukaemia virus vectors (Yu *et al.*, 1986) SIN vectors involve deletion of the U3 region of the 3' LTR. After reverse transcription and integration, the deletion is present in both proviral LTRs. This has 3 effects on the vector. Firstly, transcription from the LTR is attenuated by deletion of the major promoter elements; this prevents transcription of the full length SIN vector genome within transduced cells, ensuring a single round of infection. Secondly the reduction of transcription from the LTR increases the potential for transcriptional control using internal promoters because full-length LTRs are capable of interfering with internal promoters (Miyoshi *et al.*, 1998). Finally, LTR deletion reduces the possibility of transcriptionally activating cellular genes after proviral integration. Many SIN lentiviral vectors have been developed based on HIV-1 (Zufferey *et al.*, 1998; Miyoshi *et al.*, 1998) and efficient mobilisation of SIN vectors does not occur even in the presence of replication competent HIV (Bukovsky *et al.*, 1999). Although SIN vectors greatly reduce the chance of mobilisation, it remains possible if integration occurs in certain

contexts (Logan *et al.*, 2004). A schematic representation of reverse transcription displaying how deleting the U3 region of the 3' LTR generates 5' LTRs lacking a U3 region is displayed in figure 1.3.4.3

Much of the HIV-1 genome can be deleted to allow insertion of transgenes and promoters required for lentiviral vector-function. However, some *cis*-acting sequences are required to allow reverse transcription and packaging into retroviral particles. As well as partial LTRs, the lentiviral genome must contain a tRNA binding site (Barat *et al.*, 1989), packaging signal (Ψ) (Lever *et al.*, 1989), RRE (Daly *et al.*, 1989) and a 3' polypurine tract (PPT) (Wohrl and Moelling 1990). In addition to essential sequences, the cPPT is often retained to increase transduction efficiency (Zennou *et al.*, 2000; Sirven *et al.*, 2000). Another element often included in lentiviral vectors is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). The WPRE increases transgene expression in a cell-type independent fashion (Zufferey *et al.*, 1999) and may increase vector transcript abundance increasing the titre of a given vector (Ramezani *et al.*, 2000).

The ability to generate high titre lentiviral vectors capable of transducing nondividing cells makes lentiviral vectors a promising vector choice for gene therapy, indeed lentiviral vectors expressing antisense *env* are currently being used in clinical trials for the treatment of HIV (www.virxsys.com). After the demonstration of shRNA-mediated gene silencing, shRNA cassettes were rapidly demonstrated to be functional in lentiviral vectors (Abbas-Terki *et al.*, 2002; Rubinson *et al.*, 2003) a technology which may in the future be transferred to the clinic.

1.3.5. Limitations of lentiviral vectors and RNAi

Although initially thought to exhibit perfect target selectivity and go unnoticed by a cell's innate immune sensors, the non-specific effects of siRNAs and their precursors are becoming increasingly clear (reviewed in Sledz and Williams, 2004; Jackson and Linsley, 2004). Lentiviral vectors do not escape immune pathways and this can affect *in vitro* permissivity and transduction. Lentiviral vectors can also cause insertional mutagenesis.

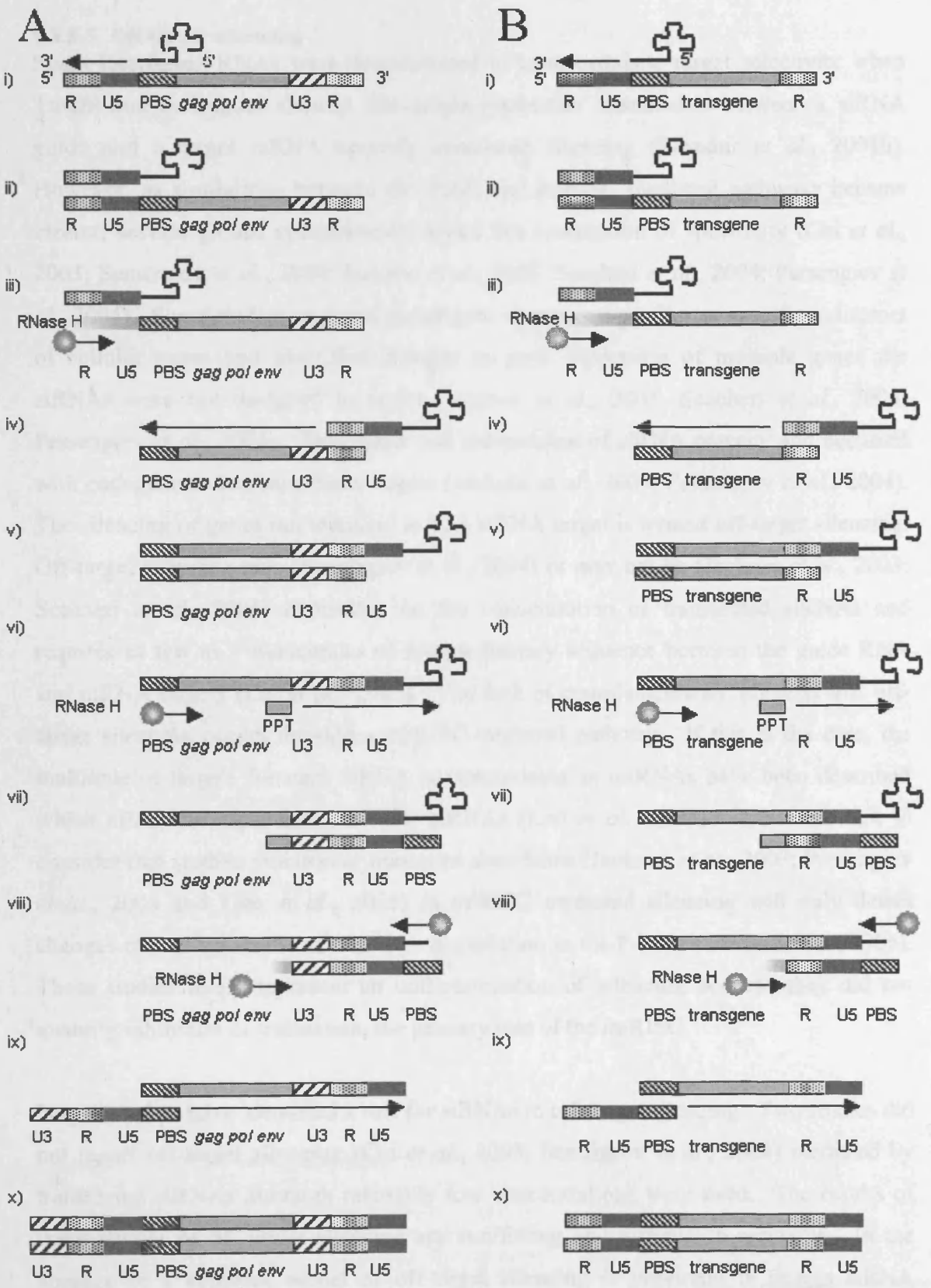


Figure 1.3.4.3. A schematic of HIV-1 and SIN lentiviral vector reverse transcription
Reverse transcription is depicted of HIV-1 (A) and a SIN lentiviral vector (B). 5'-3' DNA synthesis is indicated by arrows. SIN lentiviral vectors lacking the U3 region of the 3' LTR generate proviral DNA with no U3 region in either LTR.

1.3.5.1. Off-target silencing

Small interfering RNAs were demonstrated to have exquisite target selectivity when Tuschl and colleagues showed that single nucleotide mismatches between a siRNA guide and a target mRNA severely attenuated silencing (Elbashir *et al.*, 2001b). However, as similarities between the RISC and miRISC mediated pathways became clearer, several groups systematically tested this assumption of specificity (Chi *et al.*, 2003; Semizarov *et al.*, 2004; Jackson *et al.*, 2003; Scacheri *et al.*, 2004; Persengiev *et al.*, 2004). Some studies analysed global gene expression profiles or specific indicators of cellular stress and identified changes in gene expression of multiple genes the siRNAs were not designed to target (Jackson *et al.*, 2003: Scacheri *et al.*, 2004; Persengiev *et al.*, 2004). This effect was independent of siRNA potency and occurred with endogenous and exogenous targets (Jackson *et al.*, 2003; Persengiev *et al.*, 2004). The silencing of genes not intended to be a siRNA target is termed off-target silencing. Off-target silencing may (Persengiev et al., 2004) or may not be (Jackson *et al.*, 2003: Scacheri *et al.*, 2004) dependent on the concentration of transfected siRNAs and requires as few as 7 nucleotides of complementary sequence between the guide RNA and mRNA targets (Lin *et al.*, 2005). The lack of complementarity suggests that off-target silencing occurs through a miRISC-mediated pathway. If this is the case, the multitude of targets for each siRNA is unsurprising as miRNAs have been described which affect the expression of ~100 mRNAs (Lim *et al.*, 2005). It is important to consider that studies monitoring transcript abundance (Jackson *et al.*, 2003; Persengiev *et al.*, 2004 and Lim *et al.*, 2005) in miRISC mediated silencing will only detect changes caused by accelerated mRNA degradation in the P-body (Sen and Blau, 2005). These studies likely represent an underestimation of silencing because they did not quantify inhibition of translation, the primary role of the miRISC.

Not all studies have identified a role for siRNAs in off-target silencing. Two studies did not report off-target silencing (Chi *et al.*, 2003; Semizarov *et al.*, 2004) mediated by transfected siRNAs although relatively low concentrations were used. The results of these studies of off-target silencing are conflicting and difficult to reconcile. In the absence of a coherent model of off-target silencing is important to design siRNA experiments using the minimal effective dose of siRNAs and to consider off-target explanations for any phenotypes observed.

1.3.5.2. The cellular response to dsRNAs

The first reports of mammalian RNAi (Elbashir *et al.*, 2001a) suggested that siRNAs are too short to elicit an antiviral response in mammalian cells. Mammalian cells have many intrinsic dsRNA sensors. These sensors are integrated into the interferon response, an innate response to viral pathogens. Double stranded RNA is sensed in cells by at least 4 proteins; toll like receptor 3 (reviewed in Sen and Sarkar, 2005), the RNA

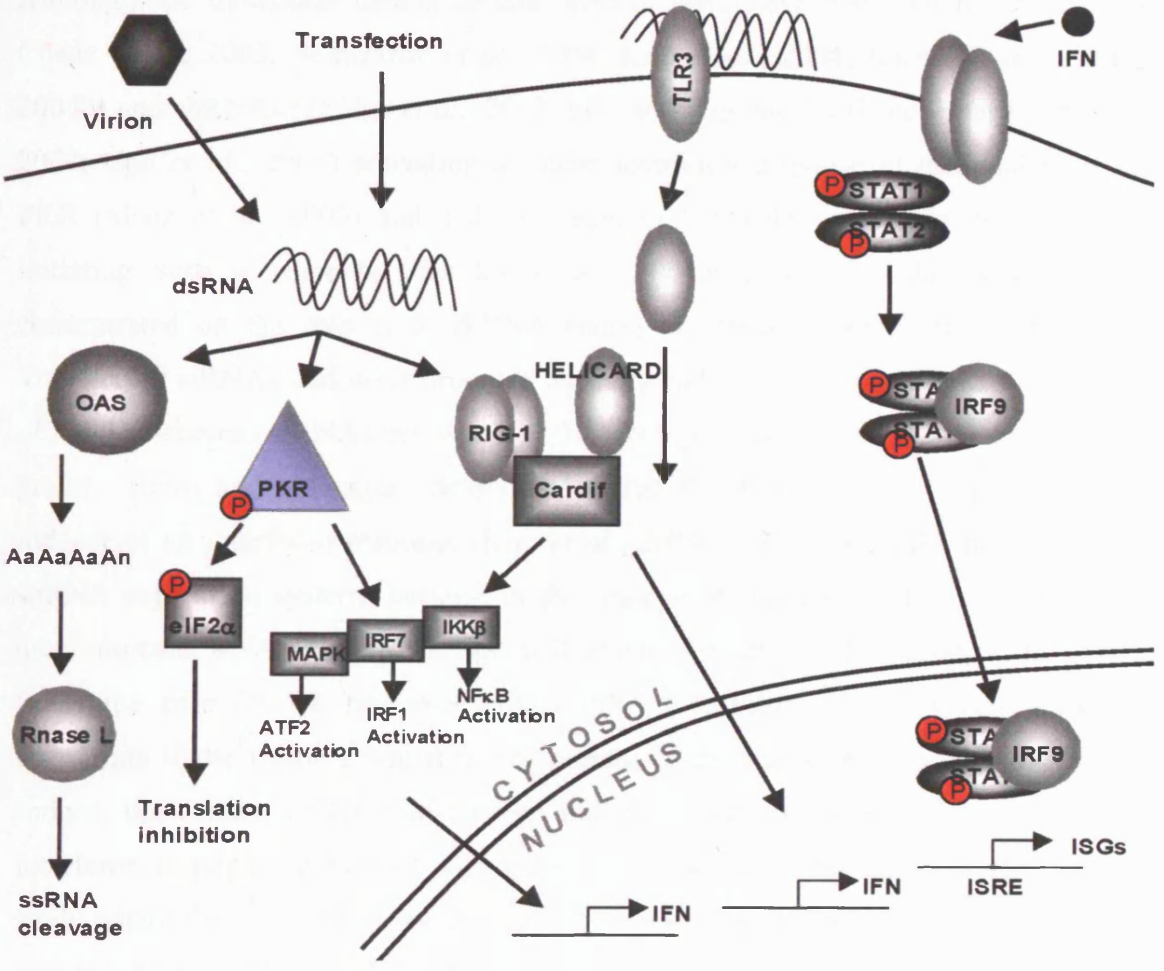


Figure 1.3.5.1. The cellular response to dsRNAs (adapted from de Veer *et al.*, 2005).
A diagrammatic representation of dsRNA activated pathways of the interferon response. The dsRNA activated protein OAS polymerises ATP 2'-5' generating oligoadenylates which activates RNase L resulting in indiscriminate RNA degradation. The dsRNA-activated kinase PKR phosphorylates eIF2α resulting in translation inhibition. PKR also initiates signal cascades resulting in interferon expression. RIG-1 and TLR-3 recognise dsRNA resulting in interferon expression. Secreted interferon leads to autocrine and paracrine activation of interferon receptors resulting in expression of interferon-stimulated genes (ISGs). IFN; interferon TLR3; Toll-like receptor 3, OAS: 2'-5' oligoadenylate synthetase, PKR; protein kinase R, eIF2α; eukaryotic initiation factor 2α, RIG-I; retinoic acid inducible gene 1, MAPK; mitogen-activated protein kinase, IRF; interferon regulatory factor, NFκB; nuclear factor kappa B; IKKβ; inhibitor of kappa B kinase beta.

helicase retinoic acid-inducible gene 1 (RIG-I) (Yoneyama *et al.*, 2004), the RNA helicase helicard (Kang *et al.*, 2002) Protein kinase R (PKR) (reviewed in Williams, 2001) and 2', 5'-oligoadenylate synthetase (OAS) whose activities are summarised in figure 1.3.5.1. PKR and OAS are the best-characterised dsRNA sensors requiring >30 bp or >65 bp of dsRNA to become activated respectively (Manche *et al.*, 1992; Minks *et al.*, 1979) comfortably above the 19 bp dsRNA in siRNAs.

Although the molecular details remain obscure there have been reports of siRNAs (Sledz *et al.*, 2003; Semizarov *et al.*, 2004; Kim *et al.*, 2004; Kariko *et al.*, 2004a; 2004b) and shRNAs (Bridge *et al.*, 2003; Fish and Kruithof 2004; Pebernard and Iggo, 2004; Cao *et al.*, 2005) activating a robust interferon response in mammalian cells. PKR (Sledz *et al.*, 2003) and Toll-like receptor 3 (TLR3) have been implicated in initiating such a response (Kariko *et al.*, 2004a; 2004b). Other studies have concentrated on the role of 5' dsRNA end(s) in activating an interferon response. Transfected siRNAs and dicer products typically end in a 5' monophosphate (Zhang *et al.*, 2002) whereas siRNAs generated by T7 RNA polymerase end in a 5' triphosphate group. Rossi and colleagues demonstrated that this triphosphate group is a potent inducer of an interferon response (Kim *et al.*, 2004). This has implications for most shRNA expression systems because in the absence of capping signals, most of which are intragenic, RNA pol III transcripts will contain a 5' triphosphate group. This seems to be the case for U6 promoter driven shRNA cassettes which express uncapped transcripts if the capping signal is not present in the transcript (Good *et al.*, 2004). Indeed, the context of U6 transcription initiation could be important in inducing an interferon response (Pebernard and Iggo, 2004) although this conflicts with another study where the authentic transcription initiation context did not prevent an interferon response (Fish and Kruithof, 2004).

No study has linked the activation of an interferon response to the potency of gene silencing. Transducing cells with lentiviral vectors differing only in shRNA sequence is capable of eliciting a robust interferon response or not eliciting a detectable response at all (Bridge *et al*, 2003). The magnitude of the interferon response also seems to be dependent on the MOI used to deliver the shRNAs (Bridge *et al*, 2003). Studies using transfected siRNAs have found that siRNA concentration also affects the interferon response (Semizarov *et al.*, 2004). Despite the importance of long dsRNA in triggering

an interferon response, no simple relationship between the shRNA length and the magnitude of an interferon response has been identified (Fish and Kruithof, 2004).

A consensus has yet to emerge on how frequently siRNAs and shRNAs induce interferon responses, and identification of common themes is confounded by the many varied techniques of introducing and generating siRNA species. Despite this, it seems clear that small dsRNAs can elicit an interferon response and poorly defined sequence and structural elements determine the extent and magnitude of a cell's response.

1.3.5.3. The cellular response to lentiviral vectors

There is a paucity of published data concerning the intrinsic cellular response to transduction with lentiviral vectors. However it is clear that vector-transduction is a less potent activator of the interferon response than shRNA expression (Bridge *et al.*, 2003). Although escaping intrinsic sensors, vector transduction can cause insertional mutagenesis (reviewed in Ferguson *et al.*, 2005). This is a particular problem with HIV-1 based vectors as integration favours transcriptionally active sites (Schroder *et al.*, 2002) and >80% of lentiviral vector insertions have been reported to occur within genes (Imren *et al.*, 2004). This represents a significant obstacle for gene therapy. Insertional mutagenesis caused by retroviral vectors (reviewed in Yi *et al.*, 2005) halted the treatment of children with severe combined immunodeficiency disease type X1 (Cavazzana-Calvo *et al.*, 2000) when three out of 11 patients developed leukaemia. To improve biosafety, many groups are trying to achieve targeted integration of lentiviral vectors (reviewed in Bushman, 2002). Although undesirable, insertional mutagenesis is not a problem in most experimental systems, but is a consideration when working with clonal transduced cell lines.

Although lentiviral vectors do not seem to be potent inducers of an interferon response, protein factors with specific antiretroviral activity are becoming increasingly well characterised (reviewed in Towers *et al.*, 2005). The recent discovery that the tripartite motif containing protein (Trim) 5α from rhesus macaque (*Macaca mulatta*) is able to attenuate HIV-1 infection (Stremlau *et al.*, 2004) exemplifies the potential complications of lentiviral transduction. Although human trim5α does not affect HIV-1, it represents a significant barrier to HIV-derived lentiviral vector transduction of non-human primate cells. It is difficult to predict the efficiency of lentiviral transduction of different cells and empirical testing is usually required. For example, lentiviral vector

stocks of a vector commonly used in our laboratory efficiently transduced EBV-transformed lymphoblastoid cell lines (LCLs), but were unable to transduce resting B-cells from which the LCLs were derived (Rosemary Tierney, personal communication). The reason for this lowered permissivity is unclear and could represent a block at any stage of transduction from viral entry to transgene expression.

1.3.6. The subjects of this thesis

An understanding of how KSHV interacts with the host cell is vital to our understanding of the viral lifecycle and of KSHV-associated transformation. We hypothesize that, similarly to EBV, KSHV has a lifecycle which is intertwined with B-cell differentiation. This thesis describes the generation of tools aimed at dissecting this relationship, revisits recombinant KSHV permissivity and identifies a possible link between B-cell terminal differentiation and KSHV reactivation.

Although extensively validated and widely available at present, lentiviral vector-mediated RNAi was unavailable when this project commenced. Chapter 3 of this thesis describes the generation and characterisation of shRNA expression cassettes that can be delivered using self-inactivating lentiviral vectors.

As proof of principle that lentiviral vector-mediated RNAi can be used to interfere with KSHV gene expression, chapter 4 of this thesis describes lentiviral vector-mediated RNA interference with KSHV ORF50 expression. Characterisation of the ORF50 'knockdown' phenotype suggests that RNAi with ORF50 expression prevents initiation of KSHV lytic replication.

The low susceptibility of B-cell lines to infection with KSHV *in vitro* is an enigmatic feature of KSHV biology. We hypothesize that *in vitro* susceptibility is related to the B-cell developmental stage and activation state of the target B-cell. Chapter 5 of this thesis describes the *in vitro* susceptibility of a panel of B-cell lines and other common cell lines to recombinant KSHV infection. Interestingly, all the adherent cell lines examined were susceptible to recombinant KSHV infection whereas we were unable to identify any B-cell lines which are efficient targets for recombinant KSHV infection.

Finally, KSHV-positive primary effusion lymphoma (PEL) cells are phenotypically similar to plasmablasts; which represent a late stage in B-cell differentiation

immediately preceding terminal differentiation into plasma cells. Chapter 6 of this thesis examines the role of XBP-1 in KSHV reactivation. Overexpressed XBP-1s is a potent activator of KSHV reactivation, driving ORF50 expression in PEL cell lines and activating the ORF50 promoter in transient-transfection reporter gene assays. This suggests that terminal differentiation, of KSHV-positive B-cells into plasma cells may be a cue for KSHV lytic reactivation.

Chapter 2

Materials and Methods

2.1. General Molecular biology Techniques

2.1.1. Preparation of competent bacteria

XL-1 Blue *Escherichia coli* (Stratagene, UK) were streaked onto Luria-Bertani (LB)-agar plates containing tetracycline (10 μg/ml) and incubated at 37°C for 16 hours. A single colony was used to inoculate 5 ml of LB-broth, supplemented with 50μg/ml tetracycline, and shaken at 37°C for 16 hours. This was added to 500 ml LB-broth and shaken at 37°C until the absorbance at 600 nm was 0.6 (around 3 hours). The culture was then put on ice to cool for 10 minutes. The bacteria were pelleted at 3000g for 10 minutes at 4°C and resuspended in 15 ml of 100 mM calcium chloride (4°C). After incubation on ice for 30 minutes, the bacteria were centrifuged again and then resuspended in 2.5 ml of 100 mM calcium chloride containing 15% glycerol by volume (4°C). The bacterial suspension was frozen on dry ice in 400 μl aliquots.

2.1.2. Introduction of plasmid DNA into *E. coli*

1-200 ng of plasmid was mixed with 200 μl of competent XL-1 Blue *Escherichia coli* (Stratagene, UK) and incubated on 1ce for 15-30 min. The cells were then heat shocked for 45 s at 42 °C and cooled on ice. 300 μl of LB broth was added to the reaction and incubated at 37 °C for 30 min. The cells were then plated on LB-agar plates containing 50 μg/ml ampicillin or kanamycin.

2.1.3. Plasmid DNA midi-preps

Midi preps of plasmid DNA were produced from 50ml overnight cultures of transformed XL-1 Blue *Escherichia coli* using the Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions.

2.1.4. Plasmid DNA mini-preps

Mini preps of plasmid DNA were produced from 3ml overnight cultures of XL-1 Blue *Escherichia coli* using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.1.5. Molecular Cloning

Taq polymerase, restriction endonucleases, mung bean nuclease, T4 polynucleotide kinase, Calf intestinal alkaline phosphatase and T4 DNA ligase were purchased from Promega, UK and were used in accordance with the manufacturer's specifications. Gel purification kits were purchased from Qiagen, UK, and were used in accordance with the manufacturer's specifications.

2.1.6. PCR cloning using the pGEM-T-Easy vector system (Promega)

The desired region was PCR-amplified using 5 units of Taq polymerase (Promega) in a 50 µl reaction volume buffered with Taq polymerase buffer A (Promega). PCR conditions involved 20-100 ng of template DNA and 30 picomoles of each oligonucleotide primer. In addition, the reaction was supplemented with a final concentration of 0.2 mM dNTPs (Promega) and between 0.5 and 2 mM $MgCl_2$ (Promega). The correct molecular weight product was separated and visualised using, Ethidium Bromide (Invitrogen) stained, agarose gel electrophoresis. The desired product was subsequently excised and extracted using the MinElute gel extraction system (Qiagen) in accordance with the manufacturer's instructions. The following ligation reaction was used. Negative controls with PCR-amplified DNA substituted with H_2O were ligated in parallel.

5 µl 2X ligation buffer
1 µl T4 DNA ligase
1 µl pGEM-T-Easy linear DNA
2 µl MinElute extracted PCR product
1 µl H_2O

Ligation reactions were transformed as described in section 2.1.2 and positive colonies were identified by blue-white-screening, PCR-screening or restriction enzyme digest as desired

2.1.7. DNA sequencing

1.5 µl of plasmid DNA was mixed with 3.5 µl of H_2O and heated to 94 °C for 5 minutes. Following incubation, 4 µl of Beckman QuickStart mix and 5 picomoles of the relevant

primer was added. The solution was placed in a thermal cycler and subjected to the following conditions:

1. 94°C 3 minutes
2. 96°C 20 seconds
3. 50°C 20 seconds
4. 60°C 4 minutes
5. Steps 1-3 an additional 29 times

DNA was precipitated from the sequencing reaction by adding 2 µl sodium acetate (1.5 M), 2 µl EDTA (50 mM, Sigma, UK) and 1 µl glycogen (20 mg/ml, Beckman Coulter, UK) in a final volume of 20 µl, the mixture was then vortexed prior to the addition of 60 µl 95% (v/v) ethanol/water (-20°C) and incubated on ice for 10 minutes. DNA was pelleted by centrifugation at 14000g for 15 minutes at room temperature. The supernatant was removed and the pellet was washed with 200 µl 70% ethanol/water (-20°C), and the tube left open at room temperature until dry. DNA was resuspended in 40 µl deionised formamide (JT Baker, USA) for 15 minutes and transferred to a 96-well plate. The sequences were determined using an automated capillary DNA sequencer (Beckman Coulter, UK) as per the manufacturer's instructions.

2.1.8. DNA oligonucleotides

Table 2.1.8. DNA primers.

Oligo Number	*Primer Name*	*Primer Sequence*
1	SJWU61-F	GGGGCTGCAGAAGGTCGGGCAGGAAGAGGGCCTATTTCCC
2	SJWU61-RC	GTTCCAATATGCATAAAAAATCTAGAGAAGCGTCGACGGTGTTTCGTCCTTTCCACAAG
3	T7	TAATACGACTCACTATAGGG
4	SP6	TATTTAGGTGACACTATAG
5	U6fillerU	TCGAATTCAGGATCCTGAGCTCGTGCA
6	U6fillerL	CGAGCTCAGGATCCTGAATTCGATGCA
7	U6 Distal	CCCATGATTCCTTCATATTTGC
8	PGEM227-207	GTTGTGTGGAATTGTGAGCGG
9	ORF73F	TTGCCACCCACGCAGTCT
10	ORF73RC	GGACGCATAGGTGTTGAAGAGTCT
11	XBP-1F	GGAAGATCTTCGGATCCCGCAATTGGTCTGGAGCTATGGTGGTGGCAGCCG
12	XBP-1RC	ATAGTTTAGCGGCCGCAACTCGAGTCAGTGGTGGTGGTGATGGTGGACACTAATCAGCTGGGGAAAGAGTTC
13	XBP-1shortF	CCTTGTAGTTGAGAACCAGG
14	XBP-1shortRC	CAGAATGCCCAACAGGATATC
15	50REDEXF	CGCTCCATTAATTGGAAGCATTCTCTCTTCATCGTGTGTGC
16	50REDEXRC	AAGCTTGCTAGCGCGGACCGCCGAAGCTTCTTACCCTAAGGAG
17	ORF50star	CACAAAAATGGCGCAAGATGACAAG
18	RedexscreenRC	ACCTTGGAGCCGTACTGGAACTGGG
19	Sjw50PROMMUTU	GCTTTTCAGGAGAGTTAGGTCGACACTGAGGATGTGGACAAGCTTCTGC
20	Sjw50PROMMUTL	GCAGAAGCTTGTCCACATCCTCAGTGTCGACCTAACTCTCCTGAAAAGC
21	SJWPrimer2076F	CGACAGATTAATCCAGGGTTTCCTGGTGGGGCGCGGC
22	SJWPrimer2276F	TCAAGCATTAATCCCGCCGGAACTCCACATCCACAAAG
23	SJWPrimer2476F	TGGGTGATTAATTCTACCACGGTCATACATTGGTGGC
24	SJWPrimer2676F	CCCCCCATTAATAGCCAGCGTATGCTTCAGGACCACC
25	SJWPrimer2876F	TCAGTCATTAATACGCTAGGGTCTCCCCACCCAACCC
26	SJWPrimer2960F	ACCGGCATTAATTTAAGCCCCGCCCAGAAACCAGTAG
27	SJWPrimer3076F	GCAGCCATTAATATGGCGCAAGATGACAAGGTAAAGATC
28	ORF50-5'	ACCGGTTCTGCTGAGAAACG
29	ORF50-3'	ACACCGTCTGTGAACTACCG

2.2. Plasmids

2.2.1. pGemU61

The human U6-promoter was PCR amplified using primers 1 and 2 using genomic DNA extracted from HBL-6 cells as a template. The PCR product was digested with *Pst* I and *Nsi* I, gel purified and ligated to *Pst* I, *Nsi* I digested self-ligated pGEM-T-Easy. The promoter was sequenced using T7 and SP6 primers.

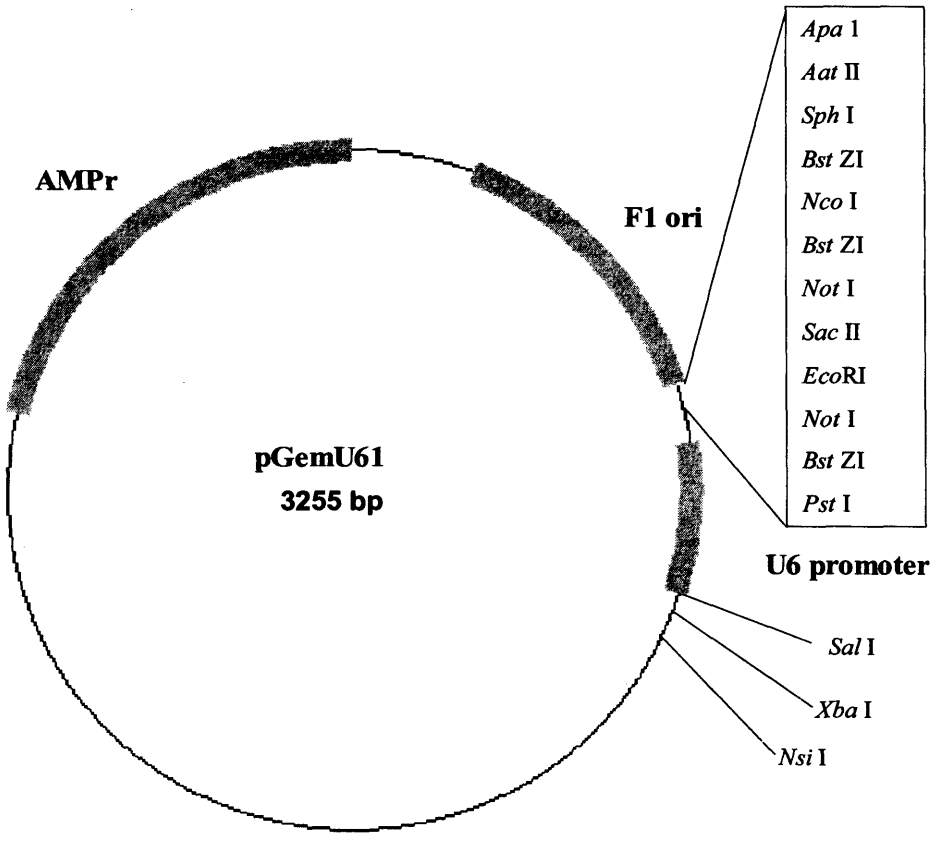


Figure 2.2.1. pGemU61
A restriction map of pGemU61 indicating the β-lactamase gene (AMPr), bacteriophage F1 origin of replication (F1 ori) and the human U6-promoter.

2.2.2. pGemU61-LINKER

Oligonucleotides 5 and 6 were annealed together, phosphorylated by treatment with T4 polynucleotide kinase and ligated to *Nsi* I digested pGEMU61. The promoter was sequenced using T7 and SP6 primers.

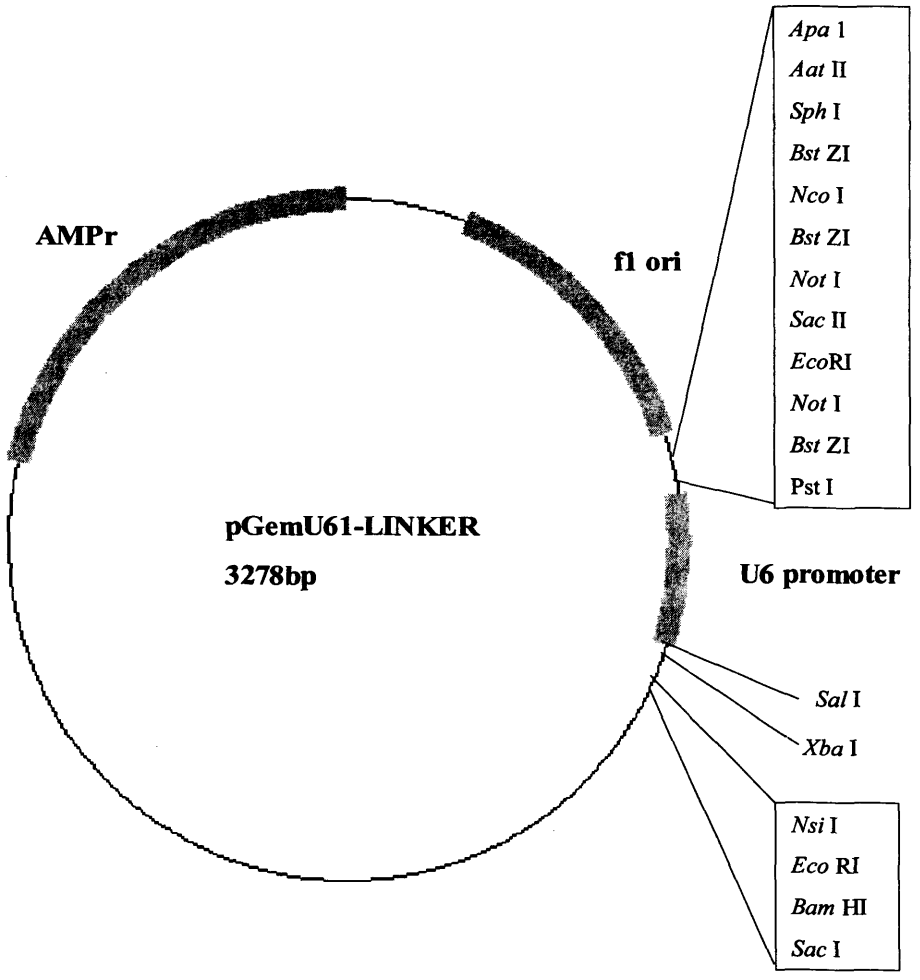


Figure 2.2.2. pGemU61-LINKER
A restriction map of pGemU61-LINKER indicating the β-lactamase gene (AMPr), bacteriophage F1 origin of replication (F1 ori) and the human U6-promoter.

2.2.3. ShRNA expressing plasmids

pGemU61-LINKER was linearised by digestion to completion with *Sal* I, blunt ended using Mung bean nuclease (Promega, UK), digested with *Xba* I and treated with Calf intestinal alkaline phosphatase (Promega, UK). The pGemU61-LINKER DNA was then ligated with complementary oligonucleotides (table 2.2.3) that had been annealed and phosphorylated through treatment with T4 polynucleotide kinase. The resulting plasmids were sequenced using oligonucleotides 7 and 8.

Table 2.2.3. Oligonucleotides used to construct shRNA expression constructs

ShRNA	*Primer Name*	*Oligonucleotide Sequence*	*Plasmid*	*Lentiviral vector*
GFP	REALSJWHPeGFPU	TTCATCTGCACCACCGGCAAGCTTCGGCTTGCC GGTGGTGCAGATGAACTTTTT	pGemU6-LINKER-RG	GFP-PAC GR1 GR2
GFP	REALSJWHpeGFPL	CTAGAAAAAGTTCATCTGCACCACCGGCAAGCC GAAGCTTGCCGGTGGTGCAGATGAA		
SJ	ORF50sjU	ATAAGGGTAGGAAGCTTTGGTGTTCGCGCCGAA GCTTCTTACCCTTGTCTTTTT	pGemU6-LINKER-50SJ	SJ-PAC
SJ	ORF50sjL	CTAGAAAAAGACAAGGGTAAGAAGCTTCGGCGC GAACACCAAAGCTTCCTACCCTTAT		
ORF50 M	ORF50mU	AAGTTCTGCAACACGTATGATATTCGTATCGTAC GTGTTGTAGAGCTTCTTTTT	pGemU6-LINKER-50M	ORF50M-PAC
ORF50 M	ORF50mL	CTAGAAAAAGAAGCTCTACAACACGTACGATACG AATATCATACGTGTTGCAGAACTT		
ORF50 E	ORF50eU	AATGTGGTGTGCAACACGTTCATTCGTGGATGTG TTGTACACCATATTCTTTTT	pGemU6-LINKER-50E	ORF50E-PAC
ORF50 E	ORF50eL	CTAGAAAAAGAATATGGTGTACAACACATCCACG AATGAACGTGTTGCACACCACATT		
TSG101	AFRtsg101F	AATCTCTAGTCTTCTTTCGTTCTTCTTCGGGACGA GAGAAGACTGGAGGTTCTTTTT	pGemU6-LINKER-Tsg101	Tsg101-PAC Tsg101-GFP
TSG101	AFRtsg101R	CTAGAAAAAGAACCTCCAGTCTTCTCTCGTCCCG AAGAACGAAAGAAGACTAGAGATT		
μ2	AFR μ2F	AGGATTAAGTGCATGGTAGGCATTCGTGCCTGC CATGCGCTTGATCTTCTTTTT	pGemU6-LINKER-μ2	μ2-PAC μ2-GFP
μ2	AFRμ2R	CTAGAAAAAGAAGATCAAGCGCATGGCAGGCAC GAATGCCTACCATGCACTTAATCCT		
Trim 5 α	Trim5a1U	CTTAGGGAGGTCAGGTTGAGTCTTCGGGCTCAA CTTGACCTCCCTGAGCTTTTT	pGemU6-LINKER-5α1	Trim5α1-PAC Trim5α1-GFP
Trim 5 α	Trim5a1L	CTAGAAAAAGCTCAGGGAGGTCAAGTTGAGCCC GAAGACTCAACCTGACCTCCCTAAG		
Blimp1 P	BLIMP1PU	AGGAGAGGTGTACATATATTGTTTCGACAATGTA TGTACACTTCTCTTCTTTTT	pGemU6-LINKER-BLIMPP	BLIMPP-PAC
Blimp1 P	BLIMP1PL	CTAGAAAAAGAAGAGAAGTGTACATACATTGTCG AAACAATATATGTACACCTCTCCT		
Blimp1 M	BLIMP1MU	GATCTCGGTGACTTTAGGAGACTTCGGTCTTCTA AAGTCATCGAGGTCCTTTTT	pGemU6-LINKER-BLIMPM	BLIMPM-PAC
Blimp1 M	BLIMP1MU	CTAGAAAAAGGACCTCGATGACTTTAGAAGACCG AAGTCTCCTAAAGTCACCGAGATC		
Blimp1 D	BLIMP1DU	AAGGTCTGACTCGAATTAATGATTCGTCATTGATT CGGGTCAGATCTTCTTTTT	pGemU6-LINKER-BLIMPD	BLIMPD-PAC
Blimp1 D	BLIMPDL	CTAGAAAAAGAAGATCTGACCCGAATCAATGACG AATCATTAATTCGAGTCAGACCTT		
PSN1	PSN1U	GTCTACTTCGTGTGTTGGTTGATTCGTCAACCAG CATACGAAGTGGACCTTTTT	pGemU6-LINKER-PSN	PSN-PAC
PSN1	PSN1L	CTAGAAAAAGGTCCACTTCGTATGCTGGTTGACG AATCAACCAACACACGAAGTAGAC		
NCN1	NSNU	AAGGGCGAGTTTCCCGTGTAGTTTCGACTGCAC GGGAAACTTGCCCTTCTTTTT	pGemU6-LINKER-NCN	NSN-PAC
NCN1	NSNL	CTAGAAAAAGAAGGGCAAGTTTCCCGTGCAGTC GAAACTACACGGGAAACTCGCCCTT		
LacZ	SJWLacZF	CTGTGATTGCGTCTGGGTTTGCTTCGGCAAGCC CGGGCGTAATCATAGCTTTTT	pGemU6-LINKER-LacZ	LacZ-PAC LacZ-GFP
LacZ	SJWLacZR	CTAGAAAAAGCTATGATTACGCCCGGGCTTGCC GAAGCAAACCCAGACGCAATCACAG		

2.2.4. Generation of lentiviral vectors encoding shRNAs

pGemU61-LINKER plasmid derivatives encoding shRNAs (table 2.2.3) were digested with *Eco*RI, gel purified and ligated to *Eco*RI linearised lentiviral vector genome plasmids (CSGW, CSRW or CSPW). The orientation of insertion was determined by *Not* I digestion, and shRNAs were sequenced using oligonucleotide 7 in their lentiviral vector context prior to use. The names of individual shRNA-expressing lentiviral vectors are listed in table 2.2.3.

2.2.5. pXBPIG

The plasmid pXBPIG was a generous gift from C. Tsantoulas who generated pXBPIG under the supervision of S. Wilson during an MSc project in the Kellam laboratory. The pXBPIG construct encodes XBP-1s tagged with a c-terminal His-tag.

2.2.6. pXBPUIG

pXBPUIG was generated through PCR amplification of XBP-1 using oligonucleotides 11 and 12. The PCR-product was cloned into pGEM-T-Easy and sequenced as described in section 2.1.7. The XBP-1u cDNA was subsequently sequenced and digested with *Bam HI* and *Xho I* and ligated to *Bam* HI and *Xho* I digested pIE, also described as CSBX, a generous gift from Y. Ikeda.

2.2.7. p50Redi

A 4099 bp region of the ORF50 promoter, first exon, intron and the first 7 codons of the second exon was PCR amplified using 100 ng of KSHV BAC36 DNA as a template. The PCR-product was cloned into pGEM-T-Easy (described in section 2.1.6). This 4099 bp fragment was then digested with *Ase* I and *Nhe* I and ligated with *Ase* I and *Nhe* I digested pCMV-DsRed-Express (Bevis and Glick, 2002) (Clontech). The construction of p50Redi is summarised in figure 2.2.6.

2.2.8. pΔ1760

To generate pΔ1760, p50Redi was digested with *Ase* I and *Nde* I. The resulting 6374 bp fragment was gel purified and self-ligated to generate pΔ1760.

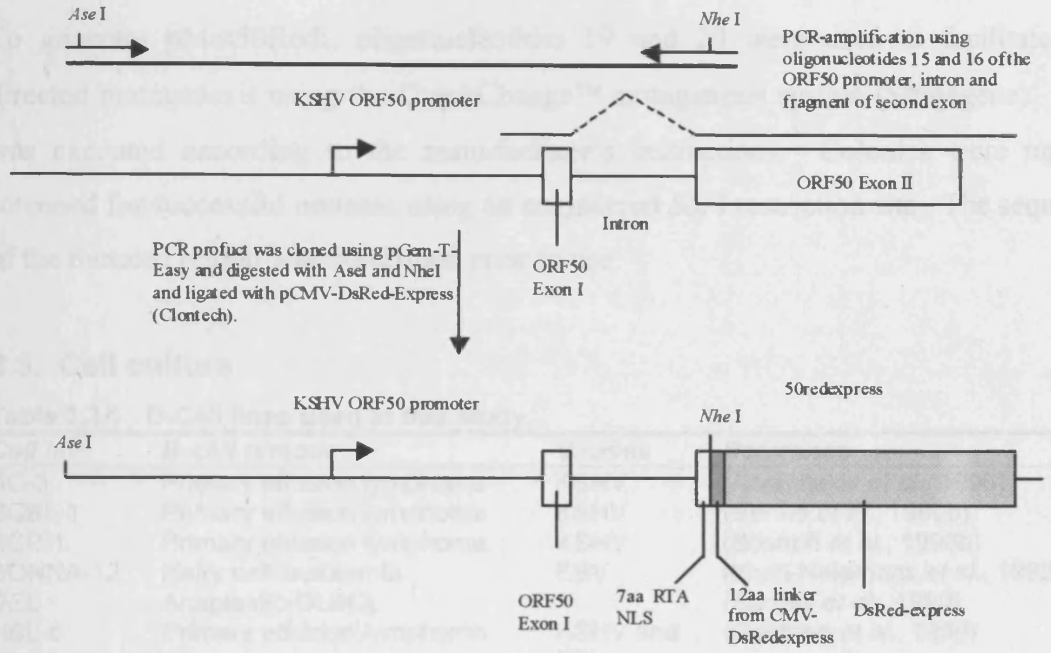
2.2.9. pNoRedi

To generate pNoRedi p50Redi was digested with *Ase* I and *Nde* I and *Nhe* I. Following digestion, the 4054 bp fragment was gel purified and the sticky ends removed through treatment with mung bean nuclease. The p50Redi plasmid was sequenced across the ligation site to confirm faithful blunt-ending and ligation had occurred.

2.2.10. pΔ2078-pΔ3076

To generate the deletion mutants of p50Redi pΔ2078 to pΔ3076, regions of the ORF50 promoter were PCR-amplified using 100 ng of KSHV BAC36 DNA as a template. Primers 21-27 were used in concert with primer 16 to PCR amplify the promoter fragments required for pΔ2078 to pΔ3076. The correct length PCR products were gel purified and cloned into pGEM-T-Easy (as described in section 2.1.6). The PCR products in pGEM-T-Easy were then digested with *Ase* I and *Nhe* I and ligated to *Ase* I and *Nhe* I digested pCMV-DsRed-Express (Clontech).

A



B

```
M  A  Q  D  D  K  G  K  K  L  R  R  S  A  L  A  L
ATG GCG CAA GAT GAC AAG GGT AAG AAG CTT CGG CGG TCC GCG CTA GCG CTA
                                                 ------
                                                 Nhe I
P  D  S  D  P  P  V  A  T  M  A  S  S
CCG GAC TCA GAT CCA CCG GTC GCC ACC ATG GCC TCC TCC
---------------------------------------
```

C

```
>RTA-Express
MAQDDKGKKLRRSALALPDSDPPVATMASSDEVIKEFMRFKVRMEGSVNG
HEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFQYGSKVYVK
HPADIPDYKKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGSFIYKVKF
IGVNFPSDGPVMQKKTMGWEASTERLYPRDGVLKGEIHKALKLKDGGHYL
VEFKSIYMAKKPVQLPGYYYVDSKLDITSHNEDYTIVEQYERAEGRHHLF
L
```

2.2.7. The construction of p50Redi

A. a diagrammatic representation of PCR-amplification, and insertion of the ORF50 promoter, first exon, intron and a 7-codon fragment of the second exon into pCMV-DsRed-Express (Clontech). B. The DNA sequence and amino acid sequence of the N-terminal region of RTA-Express (the fusion protein containing the N-terminus of RTA fused to DsRed-Express). The start codon of RTA is highlighted in red and the original start codon of DsRed-Express is highlighted in grey. The Nhe I restriction site is highlighted in blue and the coding sequence originating from the pCMV-DsRed-Express multiple cloning site is indicated with a dashed line. C. The predicted amino acid sequence of RTA-Express.

2.2.11. pMut50Redi

To generate pMut50Redi, oligonucleotides 19 and 20 were used to facilitate site directed mutagenesis using the QuickChange™ mutagenesis system (Stratagene). This was executed according to the manufacturer's instructions. Colonies were rapidly screened for successful mutants using an engineered *Sal* I restriction site. The sequence of the mutated region was confirmed prior to use.

2.3. Cell culture

Table 2.3.1. B-Cell lines used in this study.

Cell line	*B-cell tumour*	*Viruses*	*Reference*
BC-3	Primary effusion lymphoma	KSHV	(Arvanitakis *et al.*, 1996)
BCBL-1	Primary effusion lymphoma	KSHV	(Renne *et al.*, 1996b)
BCP-1	Primary effusion lymphoma	KSHV	(Boshoff *et al.*, 1998b)
BONNA-12	Hairy cell leukaemia	EBV	(Kluin-Nelemans *et al.*, 1992)
DEL	Anaplastic-DLBCL		(Barbey *et al.*, 1990)
HBL-6	Primary effusion lymphoma	KSHV and EBV	(Gaidano *et al.*, 1996)
JSC-1	Primary effusion lymphoma	KSHV and EBV	(Cannon *et al.*, 2000)
Karpas-422	Diffuse large B-cell lymphoma	EBV	(Dyer *et al.*, 1990)
L-428	Classical Hodgkin's		(Schaadt *et al.*, 1979)
NCI-H929	Multiple myeloma		(Gazdar *et al.*, 1986)
Raji	Burkitt's lymphoma	EBV	(Pulvertaft, 1964)
SK-MM-2	Plasma cell leukaemia		(Eton *et al.*, 1989)
SU-DHL-5	Diffuse large B-cell lymphoma		(Epstein *et al.*, 1978)
TOM-1	Pre-B (acute) lymphoblastic leukaemia		(Okabe *et al.*, 1987)

All cell lines are derived from B-cell tumours. Some contain the herpesviruses KSHV and/or EBV. All cell lines were grown in RPMI-1640 medium (Invitrogen, UK) with 10% foetal calf serum (FCS) with the exception of BCP-1 cells which were propagated in 20% FCS (Biowest, France). In addition, the medium was supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, UK). Cells were incubated in 5% CO_2 at 37°C.

Table 2.3.2. Cell lines, not of B-cell origin, used in this study

Cell line	*Description*	*Reference*
HeLa	epitheloid cervix carcinoma	Scherer *et al.*, 1953
HEK 293-T	293 cells containing the temperature sensitive gene encoding SV40 T-antigen	DuBridge *et al.*, 1987
SLK	Mucosal Kaposi's sarcoma lesion. From a HIV negative patient	Herndier *et al.*, 1994
MUTZ-3	Acute myeloid leukemia	Hu *et al.*, 1996
TE 671	Human medulloblastoma cell line	McAllister *et al.*, 1977
CRFK	Renal cell line established from *Felis catus*	Crandell *et al.*, 1973
FRHK	Foetal Rhesus monkey kidney cell line	Forman *et al.*, 1969
CV-1	African green monkey kidney cell line	Jensen *et al.*, 1964

All cell lines, except MUTZ-3, were grown in DMEM medium (Invitrogen, UK) with 10% foetal calf serum (Biowest, France) 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, UK) in 5% or 10% CO_2 at 37°C. SLKs were grown in medium further supplemented with1% non-essential amino acids (Invitrogen). MUTZ-3 were cultured as described by Hu and colleagues (Hu *et al*, 1996)

2.3.1. Thawing cells

Cells were removed from liquid nitrogen and thawed rapidly at 37°C. B-cell lines were added to 10 ml of Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, UK) with 10% foetal calf serum (FCS, Biowest, France), 100 units (U)/ml penicillin and 100 μg/ml streptomycin (P/S, Invitrogen, UK). The cells were then pelleted at 325 x g for 5 minutes, resuspended in 10 ml media and counted with a neubauer-improved haemocytometer. The cells were centrifuged for a further 5 minutes and resuspended at $5x10^6$ cells/ml or at the density recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). HeLa, TE 671, CRFK, FRHK, CV-1, SLK and HEK 293-T cells were added to 10 ml of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, UK) with 10% foetal calf serum (FCS, Biowest, France), 100 units (U)/ml penicillin and 100 μg/ml streptomycin (P/S, Invitrogen, UK). The cells were then pelleted at 325 x g for 5 minutes, resuspended in 10 ml media and counted with a neubauer-improved haemocytometer. The cells were plated drop-wise at the density recommended by the American Type Culture Collection (ATCC).

2.3.2. Passaging cells

Cells were cultured in RPMI-1640 with 10% FCS and P/S in 5% CO_2 at 37°C or in DMEM with 10% FCS and P/S in 5% or 10% CO_2 at 37°C.Cells were split 1:2 to 1:8, depending on cell density and rate of growth, two or three times per week.

2.3.3. Freezing cells

Cells were centrifuged at 325 x g for 5 minutes and resuspended at $5x10^6$ cells/ml in cold FCS (FCS, Biowest, France) containing 10% dimethyl sulphoxide (DMSO, Sigma, UK). Cells were aliquoted into cryovials (Nunc, USA) and gradually cooled to –80°C in an isopropanol-containing cryo-container (Nalgene, USA) before being transferred to liquid nitrogen.

2.3.4. Deriving clonal cell populations by limiting dilution

Cell density was determined using a neubauer-improved haemocytometer. Cultures were diluted to 50, 5 and 0.5 cells/ml and cultured in 96-well plates containing 200 µl/well of normal culture medium and filtered conditioned medium (1:1 ratio). The plate derived from the lowest concentration of cells that yielded viable cultures in some of the wells was selected as the source of clonal cell cultures.

HeLa C1 and JSC-1 C1 cells were generated by transduction with pHR'SINcSDW derived lentiviral vector (CSDW) using an input equivalent to a MOI 5 on HEK 293-T cells. 48-hours postinfection clonal cell populations were derived as described above. Clonal cell lines expressing dEGFP were screened by UV-microscopy and analysed by flow cytometry for a single fluorescent population, as an indication of true clonality.

JSC-1 50P and SJP lines were generated by transduction with EP and SJP lentiviral vectors with an input equivalent to a MOI 10 on HEK 293-T cells. 48-hours postinfection puromycin was added to cultures at a final concentration of 2µg/ml. 120 hours postinfection clonal cell populations were derived as described above in the continued presence of puromycin.

2.3.5. Puromycin selection following lentiviral vector transduction

48-hours postinfection the medium was removed and replaced with medium supplemented with 2 µg/ml puromycin dihydrochloride from *S. alboniger* (Sigma). The medium was removed and replaced with fresh medium supplemented with 2 µg/ml puromycin every 2-3 days until non-transduced cells, in parallel cultures, were no longer viable. The puromycin-selected cells were then cultured normally in medium supplemented with puromycin.

2.3.6. Induction of KSHV lytic replication by TPA treatment of PEL cell lines.

PEL cells were pelleted by centrifugation, and $0.5x10^6$ cells/ml were resuspended in normal culture medium supplemented with 20 ng/ml TPA (Sigma). PEL cells were incubated at 37 °C and 5% CO_2 for the duration of the experiment unless otherwise stated. Where a brief treatment of TPA was required, PEL cells were pelleted and resuspended in fresh medium and incubated at 37°C and 5% CO_2.

2.4. Lentiviral vectors

2.4.1. Transient transfection of HEK 293-T cells to make lentiviral vectors.

Lentiviral vectors were produced as described in Besnier *et al.*, 2002. HEK 293-T cells were seeded so that 10 cm dishes were confluent on the day of transfection. 18 µl of FuGENE-6 (Roche) was added to 200 µl of Opti-MEM (Invitrogen, UK). 1 µg of p8.91, 1 µg of pMDG (both generous gifts from Didier Trono) and 1.5 µg of vector-genome encoding plasmid DNA were made up to 15 µl in H_2O and added to the FuGENE-6 and Opti-MEM mixture. The transfection mixture was incubated at room temperature for 30 min before being added dropwise to the confluent HEK 293-T cells in 8 ml DMEM medium (Invitrogen, UK) with 10% foetal calf serum (Biowest, France) 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen, UK).

The HEK 293-T cells and the transfection reaction were incubated overnight at 37°C and 10% CO_2. The lentiviral vector containing supernatants were harvested at 48, 72 and 96-hours post transfection, passed through a 0.45 µm filter and stored at -80 °C in 1 ml aliquots.

2.4.2. Lentiviral vector titration of infectious units by GFP expression

$1x10^5$ HEK 293-T cells were seeded per well of a 24-well plate and cultured overnight at 37°C and 10% CO_2 in 500 µl of HEK 293-T medium (DMEM containing 10% FCS, 100 units (U)/ml penicillin and 100 µg/ml streptomycin). 80 µl, 60 µl, 40 µl, 20 µl and 1 µl of lentiviral-vector containing supernatant were mixed with HEK 293-T medium to a total volume of 100 µl. The diluted lentiviral-vector containing supernatants and 100 µl of undiluted supernatant were added to HEK 293-T cells and incubated overnight at

37°C and 10% CO_2. The cells were washed once with HEK 293-T medium and incubated for a further 36 hours.

After incubation the HEK 293-T cells were trypsinised and the percentage of GFP expressing cells determined by flow cytometry relative to non-infected cells. The percentage of GFP expressing cells was converted to infectious units by assuming a total of $2x10^5$ cells at the time of infection. Infectious units were plotted against µl of supernatant. The absolute titre of the lentiviral vector was determined by extrapolating the infectious units per ml (IU/ml) using the straight-line equation of the above plot.

2.4.3. Lentiviral vector titration of infectious units by puromycin selection and colony counting

$2x10^5$ HEK 293-T cells were seeded per well of a 24-well plate and cultured overnight at 37°C and 10% CO_2 in 500 µl of HEK 293-T medium (DMEM containing 10% FCS, 100 units (U)/ml penicillin and 100 µg/ml streptomycin). 10 µl, 1 µl, 0.1 µl, 0.01 µl and 0.001 µl of lentiviral-vector containing supernatant were mixed with HEK 293-T medium to a total volume of 100 µl. The diluted lentiviral-vector containing supernatants were added to HEK 293-T cells and incubated overnight at 37°C and 10% CO_2. The cells were then trypsinised and cultured in 5 cm dishes containing 5 ml of HEK 293-T medium containing 2 µg/ml puromycin (Sigma). 3 days after infection the plates were washed and placed in fresh HEK 293-T medium containing 2 µg/ml puromycin. 14 days after infection visible puromycin resistant colonies were counted and the infectious units extrapolated from the dilution containing approximately 10 colonies.

2.4.4. Lentiviral infection of suspension cells

1 $x10^5$ cells were pelleted and resuspended in 1 ml of the required amount of, lentiviral vector containing, HEK 293-T supernatant and culture medium. The cells were then incubated for 6 hours or overnight at 37°C and 5% CO_2. The cells were then pelleted and resuspended in fresh medium.

2.4.5. Lentiviral infection of adherent cells

1 $x10^5$ cells were plated per well of a 24 well plate. The following day the medium was removed and replaced with 1 ml of the required amount of, lentiviral vector containing,

HEK 293-T supernatant and culture medium. The cells were incubated for 6 hours or overnight at 37°C. The lentiviral vector containing, HEK 293-T supernatant and culture medium was then removed and replaced with fresh medium.

2.4.6. Spinoculation of PEL cell lines using XBPIG, IE and XBPUIG lentiviral vectors

XBPIG, XBPUIG and IE were titrated on HEK 293-T cells as described in section 2.4.2. An input equivalent to a MOI 5 on HEK 293-T cells was then used to spinoculate the required amount of PEL cells in a 24-well plate, containing $1x10^5$ cells per well, for 1 hour at 500 x g in medium supplemented with 15 μg/ml polybrene (Sigma). The cells were then incubated overnight before being pelleted and resuspended in fresh medium and passaged normally.

2.5. Flow cytometry

2.5.1. Flow cytometry

Ten thousand events were collected using a FACScan or LSR flow-cytometers with Cellquest software (Becton Dickinson, UK). Data were analysed using Windows Multiple Document Interface Flow Cytometry Application (WinMDI, J. Trotter, http://facs.scripps.edu).

In experiments analysing EmGFP and DsRed-Express expression simultaneously, data were taken using the LSR flow-cytometer. To ensure accurate quantification of EmGFP and DsRed-Express positive cells, EmGFP single expressing or DsRed-Express single-expressing controls were used in each experiment to facilitate accurate compensation. In addition, compensation between events greater than $1x10^4$ times brighter than non-transfected cells is not possible, and these cells have been removed from the analysis in chapter 6. For completeness, representative plots are included here.

In experiments simultaneously measuring intracellular RTA-staining and EmGFP expression, the LSR flow-cytometer was used. To obviate the requirement for compensation, the fluorophore allophycocyanin was always used to visualise RTA staining in EmGFP-positive cells. APC and EmGFP were excited by independent 633 nm and 488 nm light sources respectively and have non-overlaping emission absorption spectra. Compensation is therefore not required between APC and EmGFP.

2.5.2. Preparation of live cells for flow cytometry

48-hours postinfection cells were washed once with PBS, and incubated for 1 minute with trypsin-EDTA (Invitrogen) at room temperature. The trypsin was then quenched with DMEM supplemented with 10% FCS and P/S and transferred to sealed FACS tubes (Falcon). The cells were pelleted, resuspended in PBS and kept on ice prior to analysis.

2.5.3. Preparation of BAC36 infected cells for flow cytometry

See section 2.7.3.

2.5.2. Transient transfection of HEK 293-T cells with shRNA expressing plasmids

$2x10^5$ HEK 293-T cells were seeded per well of a six well plate. The following day the culture medium was removed and replaced with fresh medium. The transfection was carried out using FuGENE-6 transfection reagent (Roche) as per the manufacturer's instructions. Briefly, transfection mixes included 6 µl of FuGENE-6, 100 µl of Opti-MEM (Invitrogen) and 2.5 µg of DNA. The medium containing the transfection mixture was removed the day after transfection and replaced with fresh medium. 48-hours posttransfection live cells were analysed by flow cytometry.

2.3.3. Transient transfection of HEK 293-T cells with p50Redi and derivatives

$4x10^5$ HEK 293-T cells were seeded per well of a six well plate. The following day the culture medium was removed and replaced with fresh medium. The transfection was carried out using FuGENE-6 transfection reagent (Roche). Transfection mixes included 6 µl of FuGENE-6, 100 µl of Opti-MEM (Invitrogen) and the DNA masses listed in tables 2.3.3.1 and 2.3.3.2.

Table 2.3.3.1. DNA amounts used to transfect HEK 293-T cells in figure 6.4.1

Sample	*pCMV-RTA 6.8 kb*	*pXBPIG 11.4 kb*	*pXBPUIG 11.4 kb*	*pIE 10.3 kb*	*pBluescript II KS +*
50Redi	-	-	-	-	2.8 µg
50Redi + RTA	1.7 µg	-	-	-	1.1 µg
50Redi + IE	-	-	-	2.5 µg	0.3 µg
50Redi + XBPIG	-	2.8 µg	-	-	-
50Redi + XBPUIG	-	-	2.8 µg	-	-
50Redi+IE+RTA	0.85 µg	-	-	1.25 µg	0.7 µg
50Redi+XBPUIG +RTA	0.85 µg	-	1.4 µg	-	0.55 µg
50Redi+XBPIG+ RTA	0.85 µg	1.4 µg	-	-	0.55 µg
IE	-	-	-	2.5	1.3 µg
XBPIG	-	2.8	-	-	1 µg
XBPUIG	-	-	2.8	-	1 µg

DNA concentrations listed in addition to 1 µg of p50Redi. Last 3 samples omitted from figure 6.4.1 for clarity

Table 2.3.3.2. DNA amounts used to transfect HEK 293-T cells in figure 6.4.3

Sample	*pP50Redi or derivative*	*pXBPIG 11.4 kb*	*pIE 10.3 kb*	*pBluescript II KS +*
P50Redi+pIE	1 µg	-	2.5 µg	0.3 µg
P50Redi+XBPIG	1 µg	2.8 µg	-	-
pΔ1760+ pIE	1 µg	-	2.5 µg	0.3 µg
pΔ1760+ pXBPIG	1 µg	2.8 µg	-	-
pΔ2078-pNoredi + pIE	1 µg	-	2.5 µg	0.3 µg
pΔ2078-pNoredi + pXBPIG	1 µg	2.8 µg	-	-
P50Redi	1 µg	-	-	2.8 µg
pIE	-	-	2.5 µg	1.3 µg
pXBPIG	-	2.8 µg	-	1 µg

Last 3 samples omitted from figure 6.4.3 for clarity

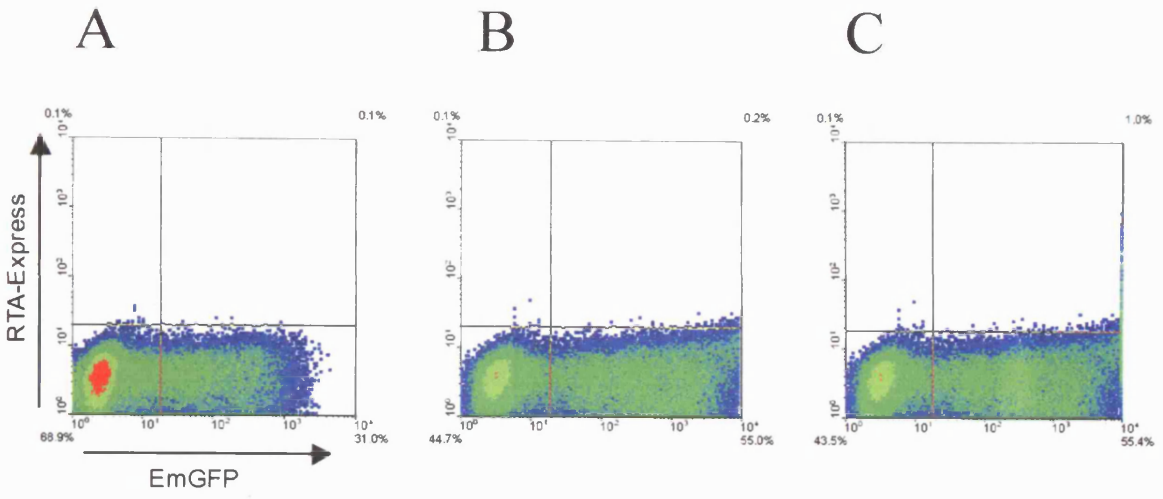


Figure 2.3.3. EmGFP controls in p50Redi reporter gene experiments
A. Typical fluorescent control of pXBPIG transfected in the absence of RTA-Express encoding plasmids. B. Typical fluorescent control of pIE transfected in the absence of RTA-Express encoding plasmids. C. The density plot in B is shown prior to the removal of extremely bright events. The data in figures 6.4.1 and 6.4.3 also has the brightest events removed.

2.5.3. Intracellular antigen staining

$5x10^5$ cells were fixed with 4% paraformaldehyde (Sigma) for 10 minutes on ice before quenching with a 10-fold excess of PBS supplemented with 5% glycine (Sigma). Cells were washed once in wash buffer (PBS supplemented with 2% FCS and 0.01% sodium azide) before being resuspended in permeabilisation buffer (PBS supplemented with 2% FCS, 0.1% Triton-X-100 and 0.01% sodium azide). Antibody incubations were performed in either 10 µl or 50 µl volumes of permeabilisation buffer further supplemented with 10% FCS. The cells were then washed 3 times with permeabilisation buffer subsequent to either antibody incubations or a final wash with wash buffer prior to data collection.

2.5.4. Nuclear staining for KSHV ORF50 in cells not expressing Emerald

The anti-RTA antibody (supernatant mouse mAb, IgG a generous gift from Keiji Ueda) was used diluted 1:2000 with control mouse IgG (a generous gift from Stuart Neil) used at an equivalent concentration as an isotype control. The secondary antibody for indirect immunofluorescence was tetramethylrhodamine isothiocyanate (TRITC)-conjugated polyclonal rabbit anti-mouse IgG (DAKO R0270) used diluted 1:400.

2.5.5. Nuclear staining for KSHV ORF50 in cells expressing Emerald

The anti-RTA antibody was used diluted 1:500000 with control mouse IgG used at an equivalent concentration as an isotype control. The secondary antibody for indirect immunofluorescence was polyclonal goat anti-mouse IgG conjugated to allophycocyanin (BioLegend) used diluted 1:400.

2.5.6. Intracellular staining for ORF K8

The anti-K8 antibody (supernatant mouse mAb IgG a generous gift from Keiji Ueda) was used diluted 1:2000 with control mouse IgG (a generous gift from Stuart Neil) used at an equivalent concentration as an isotype control. The secondary antibody for indirect immunofluorescence was tetramethylrhodamine isothiocyanate (TRITC)-conjugated polyclonal rabbit anti-mouse IgG (DAKO R0270) used diluted 1:400.

2.5.7. Intracellular staining for ORF59

The anti-ORF59 antibody (purified mouse mAb IgG_{2b}, Advanced Biotechnologies inc.) was diluted 1:100 with control mouse IgG_{2b} (a generous gift from Stuart Neil) used at an equivalent concentration as an isotype control. The secondary antibody for indirect immunofluorescence was tetramethylrhodamine isothiocyanate (TRITC)-conjugated polyclonal rabbit anti-mouse IgG (DAKO R0270) used diluted 1:400.

2.6. Detecting virion-associated genome copies using quantitative PCR

2.6.1. Induction of lytic replication

JSC-1 cells and JSC-1 cells transduced with lentiviral vectors RP and EP were pelleted and resuspended at a density of $1x10^5$ cells/ml or $5x10^4$ cells/ml in 10 ml in 15 ml falcon tubes. For time courses 300 µl was removed and used as the t=0 sample. TPA was added directly to the medium and cell mixture at a final concentration of 20 ng/ml (Sigma, dissolved in DMSO at 0.2 mg/ml). The TPA containing cell suspension was mixed thoroughly and then incubated for 1 hour at 37°C and 5% CO_2. The cells were then pelleted and resuspended in fresh medium and incubated at 37°C and 5% CO_2.

2.6.2. DNA extraction

300 µl of cells and culture medium was harvested. Cells were removed by centrifugation at 500 x g for 5 minutes and 200 µl of cell free supernatant was removed.

To remove contaminating episomal DNA 1 µl of a 1M $MgCl_2$ solution (Sigma) and 1 µl (10 units) of DNase 1 (Roche) was added to the cell free supernatant and incubated at 37 °C for 1 hr. DNA was subsequently extracted using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

2.6.3. Quantitative PCR ORF73 standards

A linearised DNA fragment, encoding KSHV ORF73 and *H. sapiens* Glyseraldehyde-3-phosphate dehydrogenase (a generous gift from Dimitra Bourboulia), at $1x10^8$ copies/µl was serially diluted to form plasmid standards of $1x10^5$, $1x10^4$, $1x10^3$ and $1x10^2$ copies/µl. 10 µl was used of each of these standards giving a range of $1x10^6$, $1x10^3$ copies to form the standard curves.

2.6.4. Quantitative PCR

Based on a protocol developed by Dimitra Bourboulia (Bourboulia *et al.*, 2004) DNA oligonucleotides 9 and 10 were used to amplify KSHV ORF73 using the probe 5'-6-FAM-TCTTCTCAAAGGCCACCGCTTTCAAGTC-TAMRA-3'. A master mix was prepared in a plasmid/cDNA free environment using ABsolute™ QPCR ROX mix (ABgene). The master mix was prepared in the volumes listed below per well.

25 µl ABsolute™ QPCR ROX mix
3.5 µl primer 9 (10 µM)
3.5 µl primer 10 (10 µM)
1.5 µl probe (5 µM)
6.5 µl H_2O

The master mix was vortexed for 10 s and transferred to ABgene Thermo-Fast® 96-well PCR plates (40 µl/well). The plate was then covered and moved out of the plasmid/cDNA free environment and 10 µl/well of plasmid standard, H_2O control or extracted DNA was added. The plate was covered with an optical adhesive cover (ABgene). The PCR was executed using an ABI Prism 7000 (Applied Biosystems) using the standard hot start cycling parameters.

1. 10 min 95 °C
2. 1 min 95 °C
3. 1 min 60 °C
4. Steps 2-3 an additional 39 times

2.6.5. Quantitative PCR data analysis

The data were analysed using ABI prism 7000 SDS software CTS 1.0 (Applied Biosystems). Using the standard curves in figures 2.6.5.1 and 2.6.5.2 absolute copies of ORF73 present in the PCR reaction could be generated. These values were then multiplied by 6 (elution volume from DNA extraction was 60 µl), and then multiplied by 5 (DNA was extracted from 200 µl of tissue culture supernatant) to give a value of copies/ml. All PCR reactions were carried out using triplicate repeat amplifications and the data were only accepted if no copies were detected in H_2O controls or in DNA extractions from medium prior to contact with cells containing $1x10^6$ copies of the ORF73 standard (indicating successful DNase treatment). In figure 4.2.4 A the copy number was then divided by the number of cells/ml determined using triplicate counts on a Neubauer improved haemocytometer to give a value of copies/cell

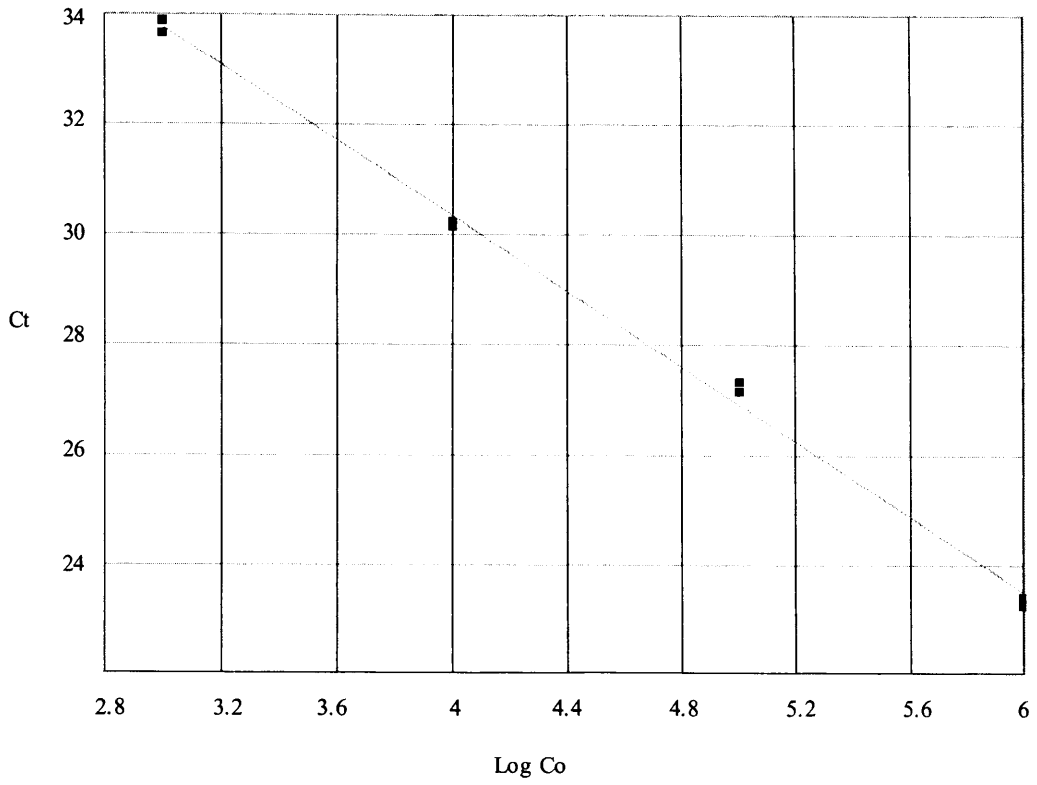


Figure 2.6.5.1. Standard curve for figure 4.2.4 A.
Standard Curve for the data presented in figure 4.2.4 A. R^2 : 0.996981. Generated by ABI prism 7000 SDS CTS 1.0.

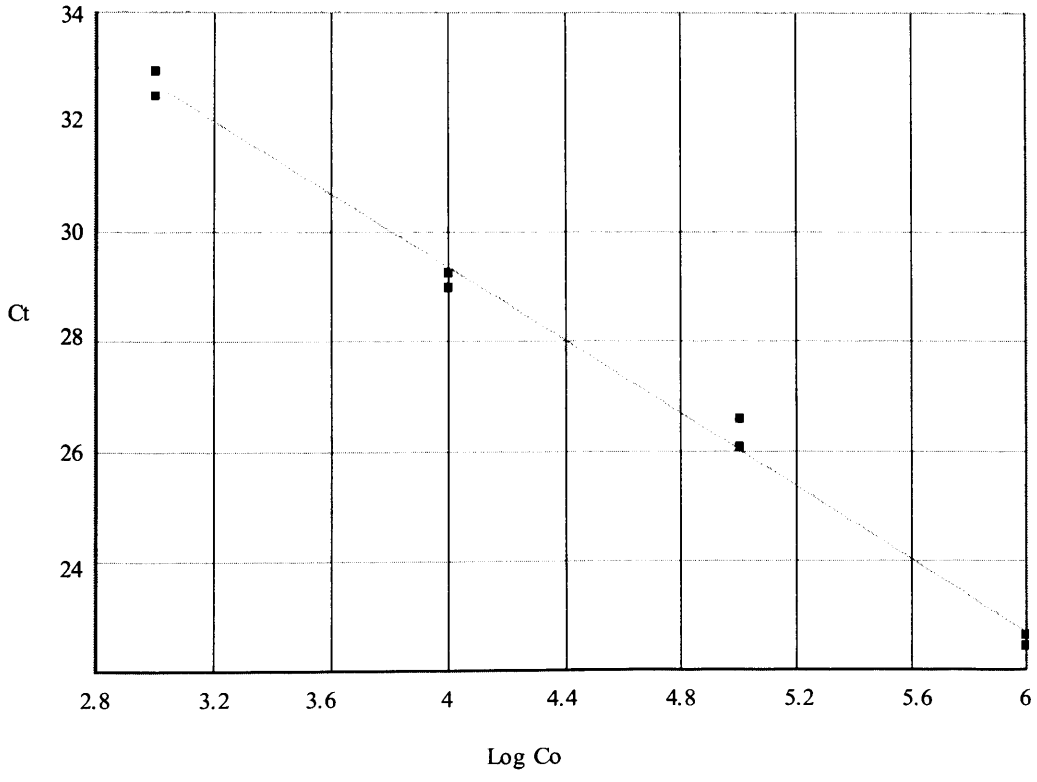


Figure 2.6.5.2. Standard curve for figure 4.2.4 B.
Standard Curve for the data presented in figure 4.2.4 B. R^2 : 0.994255

2.7. KSHV BAC36

2.7.1. BAC DNA extraction

KSHV BAC36 was a generous gift from S. J. Gao. Isolation of BAC DNA was achieved using a Plasmid Midi Kit (Qiagen) using the-user developed protocol (http://www1.qiagen.com/literature/protocols/pdf/QP01.pdf). Bacteria were propagated using LB-agar plates or LB-broth containing 5 μg/ml chloramphenicol.

2.7.2. Establishing BAC36 producer HEK 293-T cell lines

$2x10^5$ HEK 293-T cells were transfected as described above (2.5.2) using 1 μg of BAC36 DNA, 6 μl of FuGENE 6 (Roche) and 100 μl of Opti-MEM (Invitrogen). 48-hours posttransfection cells were trypsinised and transferred to 10 cm plates (Techno Plastic Products AG) and expanded in DMEM with 10% FCS and P/S supplemented with 200 μg/ml Hygromycin B changing the medium once every week. 4 weeks posttransfection, the medium was changed and replenished with fresh medium containing TPA at a final concentration of 20 ng/ml and incubated for 1 hour at 37°C and 10% CO_2. The medium was then removed and replaced with fresh, hygromycin free, medium and incubated at 37°C and 10% CO_2 for 6 days. The KSHV BAC36 containing supernatant was harvested, passed through a 0.45 μm filter and stored at -80 °C in 1 ml aliquots.

KSHV BAC36 containing supernatant was thawed on ice and 15 μg of hexadimetrine bromide (polybrene, Sigma) was added and mixed thoroughly. 1 well of a 24-well plate (TPP) containing $1x10^5$ HEK 293-T cells/well was infected through spinoculation at 500 x g for 1 h at room temperature, using a Legend RT centrifuge (Sorvall), with 1ml of undiluted KSHV BAC36 containing supernatant containing 15 μg polybrene. 48-hours postinfection cells were trypsinised and transferred to 10 cm plates (TPP) and expanded in DMEM with 10% FCS and P/S supplemented with 200 μg/ml Hygromycin B changing the medium once every week. Two weeks postinfection 11 hygromycin resistant colonies were removed using a 2 ml pipette and propagated independently

2.7.3. Production and harvesting of infectious KSHV BAC36

$1x10^7$ KSHV BAC36 infected HEK 293-T cells were seeded in 10 cm plates (TPP) and expanded in DMEM with 10% FCS and P/S supplemented with 200 μg/ml Hygromycin

B. The following day, the medium was removed and replaced with medium containing TPA at a final concentration of 20 ng/ml and incubated for 1 hour at 37°C and 10% CO_2. The medium was then removed and replaced with fresh, hygromycin free, medium and incubated at 37°C and 10% CO_2. The KSHV BAC36 containing supernatant was harvested, passed through a 0.45 μm filter and stored at -80 °C in 1 or 10 ml aliquots.

2.7.4. Titration of infectious KSHV BAC36

KSHV BAC36 containing supernatant was thawed on ice and 15 μg of hexadimetrine bromide (polybrene, Sigma) was added per ml of supernatant and mixed thoroughly. DMEM supplemented with 10% FCS and P/S was also mixed with polybrene and used as diluent. Several dilutions of each supernatant were prepared and 1 well of a 24-well plate (TPP) containing $1x10^5$ HEK 293-T cells/well was infected for each dilution through spinoculation at 500 x g for 1 h at room temperature, using a Legend RT centrifuge (Sorvall). 48-hours postinfection cells were trypsinised and transferred to sealed FACS tubes (Falcon). The cells were washed once in PBS, fixed in 3.3% formaldehyde (TAAB) for 10 minutes on ice before quenching with a 10-fold excess of PBS supplemented with 5% glycine (Sigma). The cells were pelleted and resuspended in PBS and the proportion of EGFP-positive cells was determined by flow cytometry. The percentage of GFP expressing cells was converted to infectious units by assuming a total of $2x10^5$ cells at the time of infection. Infectious units were plotted against μl of supernatant. The absolute titre of recombinant KSHV was determined by extrapolating the infectious units per ml (IU/ml) using the straight-line equation of the above plot.

2.8. Confocal microscopy

Images of GFP fluorescence were gathered using an Axiovert 100 TE microscope (Zeiss) and Lasersharp 2000 (Bio-Rad).

Imaging of HeLa C1 cells following transduction with GP was achieved by trypsinising puromycin resistant cells 144 hours post-transduction and re-seeding them on coverslips. 168 hours post-transduction, the medium was removed, cells fixed in 4% paraformaldehyde (Sigma) and quenched in PBS supplemented with 5% glycine (Sigma). Coverslips were washed twice in PBS and once in water before mounting on slides using mowiol (Sigma). Destabilised GFP expression was visualised through

excitation with 488 nm light of the equivalent intensity for each sample. No image enhancement was used. HeLa cell images in figure 3.2.3 are magnified 20 times.

Imaging of live chondrocytes was achieved through replacing the growth medium with PBS. EGFP expression was visualised through excitation with 488 nm light of the equivalent intensity for each sample. No image enhancement was used. The chondrocyte images in figure 5.2.3 are magnified 40 times.

2.9. Determination of XBP1 splice status by RT-PCR

2.9.1. RNA extraction and DNase treatment

RNA was extracted using the TRIzol reagent (Invitrogen). In accordance with the manufacturer's instructions. Briefly, $5x10^6$-$1x10^7$ cells were lysed per ml of TRIzol reagent. The cell lysate was then incubated at 70 °C for 10 min prior to repeated chloroform extraction. The RNA was precipitated using isopropanol followed by washing of the pellet with 75% Ethanol. The pellet was air dried and resuspended in RNase free water prior to DNase treatment.

DNase treatment was executed using RQ1 RNase-free DNase (Promega) in accordance with the manufacturer's instructions. Following treatment, the reaction was extracted twice using phenol:choloroform:isoamylalcohol (25:24:1) (Sigma). The RNA was then precipitated using 0.2 volumes of 8M Ammonium acetate (Sigma) and 2.5 volumes of 95% ethanol. The pellet was then washed in 75% ethanol prior to air-drying and resuspension in RNase-free H_2O.

2.9.2. cDNA synthesis

Reverse transcription reactions were carried out using Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) Promega, in accordance with the manufacturer's instructions.

2.9.3. XBP1 PCR, *Pst* I digestion and resolution

PCR amplification across the XBP-1 atypical splice junction was achieved using oligonucleotides 13 and 14. The desired region was PCR-amplified using 5 units of Taq polymerase (Promega) in a 50 µl reaction volume buffered with Taq polymerase buffer

A (Promega). PCR conditions involved 20-100 ng of template plasmid DNA or 2 µl of cDNA and 30 picomoles of each oligonucleotide primer. In addition, the reaction was supplemented with a final concentration of 0.2 mM dNTPs (Promega) and 1 mM $MgCl_2$ (Promega). The PCR was executed using the following cycle:

1. 30 Seconds at 95 °C
2. 30 seconds at 55 °C
3. 1 min at 72 °C
4. Steps 1-3 an additional 39 times
5. 2min at 72 °C

Following PCR amplification, 2 µl of H_2O or 20 units of *Pst* I was added to each reaction, which was incubated overnight at 37 °C. 2 µl of 6X loading buffer (Fermentas) was then added to 10 µl of each reaction and resolved using agarose gel electrophoresis. The best separation was achieved using pre-cooled 3 % TBE buffered agarose gels, pre-cooled to 4 °C. The gels were resolved at 5 volts/cm in room temperature TBE buffer diluted 1:1 with H_2O.

2.9.4. DTT treatment of PEL cells

To induce an UPR, $5x10^7$ JSC-1 and BC-3 PEL cell lines were cultured in normal medium or medium containing 2 M DTT. Following treatment, RNA was extracted as described in section 2.9.1.

2.10. PCR-amplification and sequencing of the shRNA-50E target sequence

DNA was isolated from PEL cell lines using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. 20ng of DNA was used to PCR-amplify the target region of shRNA-50E using oligonucleotides 28 and 29 and the following PCR cycle.

1. 30 Seconds at 95 °C
2. 30 seconds at 55 °C
3. 1 min at 72 °C
6. Steps 1-3 an additional 29 times
7. 2min at 72 °C

The PCR-product was cloned using the pGEM-T-Easy kit (Promega) as described in section 2.1.6 and 6 positive clones from each cell line were sequenced as described in section 2.1.6.

2.11. Microarray analysis

2.11.1. RNA extraction

RNA was extracted as in section 2.9.1.

2.11.2. Labelling (performed by Dr Catherine Gale)

A direct incorporation method (CyScribe First-Strand cDNA Labelling Kit) was used (Amersham Biosciences, GE HealthCare). Specifically, 1 µl oligo(dT) and 3 µl KSHV-specific primers were added to 25 µg total RNA (TRNA) in a 0.5 ml PCR tube on ice. The reaction mixture was incubated at 70°C for 5 minutes followed by incubation at room temperature for 10 minutes. Subsequently, 4 µl of CyScript buffer, 2 µl 0.1M DTT, 1 µl dCTP nucleotide mix, 1 µl dCTP CyDye-labelled nucleotide and 1 µl CyScript reverse transcriptase were added to the reaction mixtures. These were then incubated at 42°C for 1.5 h. On all occasions, reference RNA (batched mixture of TPA-induced JSC-1 and HeLa TRNA) was labelled with Cy3 labelled nucleotide and experimental sample with Cy5. The RNA was then denatured by addition of 2.5 µl 0.5 M EDTA (pH 8.0) and 10 µl 0.1 M NaOH and incubated at 70°C for 10 minutes, the reaction neutralised by addition of 10 µl 0.1 M HCl, 3 µl CoT-1 DNA (Gibco), and 450 µl TE (pH 8.0). Labelled RNA was purified using a Microcon-100 filter and reduced to 12 µl. The labelled RNA was immediately prepared for hybridisation.

2.11.3. Hybridisation (performed by Dr Catherine Gale)

The Cy3 and Cy5 labelled probes were made up to 14 µl with TE (pH 8.0) and the hybridisation mixtures prepared as follows: 12 µl 20x SSPE (Sigma), 1.1 µl 0.5 M EDTA, 2 µl poly dA_{40-60} (Pharmacia), 2 µl yeast tRNA (Sigma), 14 µl Cy3 probe, 14 µl Cy5 probe, and finally 1 µl 10% SDS. The mixture was vortexed and incubated at 98°C for 2 minutes followed by 37°C for 20 minutes. Subsequently 1µl 100x Denhardt's solution (Sigma) was added to the probe mixture and centrifuged at 13,000 rpm for 15 minutes at RT. Arrays previously printed in-house (a generous gift from C. Li) and baked at 70°C for 3+ hours to immobilise probes were blocked prior to hybridisation. Blocking solution consisted of an aqueous solution of 5x SSC, 0.1 % SDS, 0.05 % BSA and 0.05 % Milk. This was warmed to 50°C and passed through a 0.45 µm filter prior to blocking, which was achieved by first immersing the arrays in blocking solution for 45 min at 50°C. Arrays were then rinsed twice thoroughly with purified water, incubated in water (95°C) for 2 min, and the first blocking procedure repeated. Arrays were rinsed a second time, soaked in isopropanol for 30 sec, then immediately dried by centrifugation at 350 x g for 2 minutes at room temperature. Arrays were then either stored in moisture-free environment for use within 4 hours, or were transferred directly to hybridisation chambers, preheated to 65 °C, and allowed to warm for 30 minutes. Glass cover slips were cleaned by immersion in 95% ethanol for 2 minutes and centrifugation at 350 x g for 2 minutes to dry. 46 µl of the probe was applied to the array by adding it to the glass coverslip, this was then applied to the pre-warmed array by gently lowering the array over the coverslip until capillary action adhered the two together and distributed the probe, with no need for application of pressure. The array was placed back into the hybridisation chamber and 150 µl 4x SSPE (65°C) added, the chamber lid was firmly secured and the assembly transferred to a 65°C water bath and incubated for 16 h.

After incubation the hybridisation chamber was dismantled at 65°C and the array carefully removed and immersed in 2x SSPE heated to 50°C. Once the coverslip had dissociated the array was immersed in fresh 2x SSPE for 2 minutes at RT (rolling), followed by 1x SSPE for 2 minutes and 0.1x SSPE for 3 minutes. Subsequently, the array was transferred to a clean 50 ml Falcon tube and centrifuged at 350 x g for 2 minutes to dry.

2.11.4. Array scanning (performed by Dr Catherine Gale)

Arrays were scanned at 10 μm resolution using the GenePix 4000B array scanner (Axon instruments, Molecular Dynamics) and the images analysed using GenePix Pro 3.0 software. Cy3 and Cy5 fluorophores were simultaneously excited at 532 nm and 635 nm. A template was fitted over the array image using GenePix Pro 3.0. All the elements on each array were checked by eye and manually corrected where necessary. Data were extracted from the image by using the adjusted template and the normalisation factor (average ratio between signals in the Cy3 and Cy5 channels) calculated automatically by the GenePix software. Expression ratios were calculated as the median of the ratios between the local background-subtracted Cy3 and Cy5 signals on a pixel-by-pixel basis. The data were exported to a spreadsheet created in Excel. The median of ratios were filtered to remove flagged array elements and elements for which the signal to background ratio was below a stringent threshold in both Cy5 and Cy3 channels.

2.11.5. Array analysis

2.11.5.1. Cluster and treeview (performed by Dr Catherine Gale)

The final array data were analysed using Cluster software (Eisen *et al.*, 1998). The program Cluster assembles a set of elements (genes or arrays) into a tree, where elements are joined by tree branches whose length is proportional to the distance (correlation coefficient) between the elements. Array elements for which expression measurements failed, the signal to noise ratio filter were removed and the data converted to log base 2. The arrays and genes were then median centred (the median expression ratio within each array or of each array element across all arrays was set to 0). A self-organising map algorithm was then applied, in order to direct the orientation of nodes generated by hierarchical clustering. Genes and/or arrays were clustered by average-linkage hierarchical clustering and the results were visualised with the software Treeview.

2.11.5.2. Significance analysis of microarrays (performed by Dr Catherine Gale)

SAM (Significance Analysis of Microarrays) is a statistical technique for finding significantly differently expressed genes in a set of microarray experiments (Tusher *et al.*, 2001) and functions as a software package (written by B. Narasimhan) for Excel. The input to SAM is a gene expression matrix from a set of microarray experiments, as well as a response variable from each experiment. In the case of these experiments, the response variable is uninduced JCS-1 vs. TPA-treated JSC-1, IE-transduced, XBPUIG-transduced or XBPIG-transduced. By inputting normalised log2 ratios (>4 replicates

for each array/response variable) SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific T-tests. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores greater than a threshold are deemed differentially expressed. The percentage of such genes identified by chance is calculated by a false discovery rate (FDR). To estimate the FDR, genes are randomly permuted in a form of bootstrapping. The FDR threshold can be adjusted to identify smaller or larger sets of genes. The FDR for these experiments was selected as 5%.

Chapter 3

Results: Lentiviral vector-mediated RNAi

3.1. Generating a functional shRNA cassette

Although the use of lentiviral vectors to deliver shRNAs is now commonplace, when the work described in this chapter commenced such an approach had not been reported and no vectors were available. We wanted to use RNAi in PEL cell lines to study host-virus interactions. Because PEL cell lines are difficult to transfect, we wanted to modify lentiviral vectors to deliver shRNA expression cassettes capable of mediating RNAi.

3.1.1. pGemU61

To generate an shRNA vector-system capable of gene silencing in human cell lines we generated pGemU61 (Figure 2.2.1). This plasmid contains the human U6-promoter which was previously shown to express shRNAs and achieve gene silencing (Paul *et al.*, 2002; Paddison *et al.*, 2002). The U6-promoter was cloned from the human cell line HBL-6 and shares 100% sequence identity to nucleotides 1-267 of the human U6-promoter (accession number: X07425).

PGemU61 was designed to facilitate the insertion of shRNA coding sequences, whilst preserving the authentic sequence of the U6-promoter. To achieve this the restriction endonuclease recognition sites *Sal* I and *Xba* I were introduced downstream of the U6-promoter during PCR amplification (detailed in materials and methods). These sites allow insertion of synthetic oligonucleotide-derived sequences encoding shRNAs (Figure 3.1.1). The *Sal* I restriction site is downstream of the U6 transcription start site, potentially encoding transcribed nucleotides. We wished to generate a vector that did not encode superfluous sequence proximal to the shRNA duplex. To prevent undesired sequence, originating from the *Sal* I restriction site, from being expressed the 5' overhang resulting from *Sal* I digestion can be removed by mung bean nuclease treatment, generating a blunt DNA end. Subsequent *Xba* I digestion generates an *Xba* I 'sticky end' facilitating directional cloning of annealed oligonucleotides encoding shRNAs. The 5' G is retained after mung bean nuclease treatment allowing DNA sequences to be inserted into pGemU61 encoding any desired RNA gene initiating with guanine.

To avoid undesired sequence at the 3' end of RNA transcribed from pGemU61, an RNA polymerase III termination sequence is inserted 5' of the *Xba* I site in the shRNA oligonucleotides. This results in transcription termination prior to sequences encoded by the *Xba* I site. A second termination sequence (TTTTTT) is present in pGemU61 downstream of the *Xba* I site to facilitate insertion of genes lacking a termination sequence.

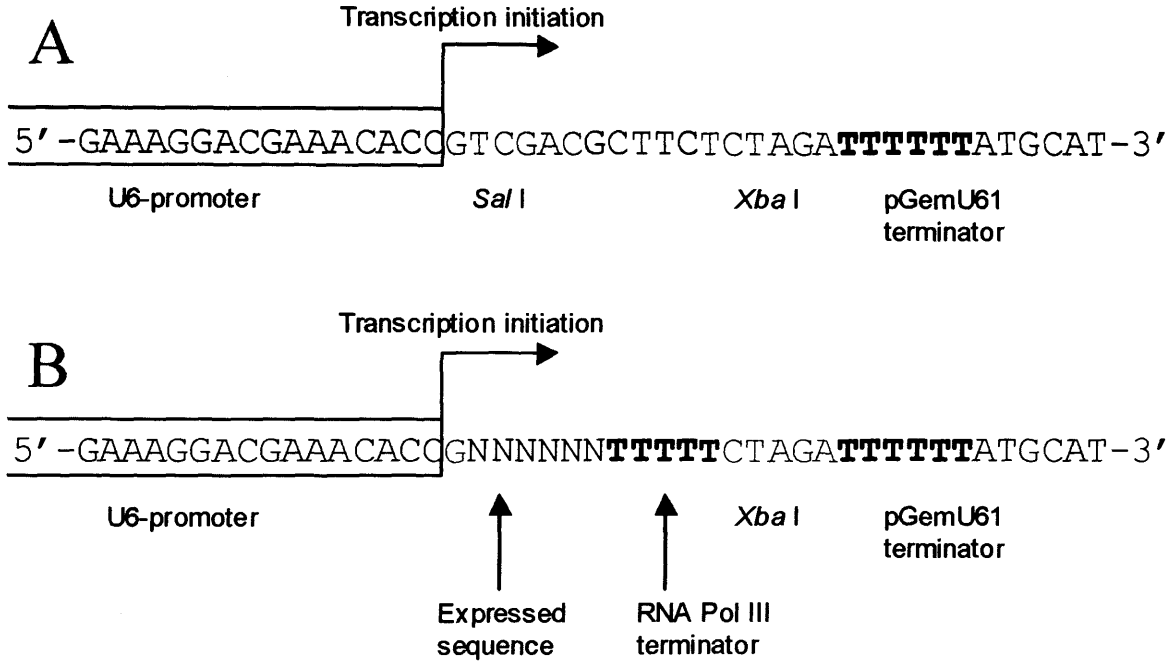


Figure 3.1.1. The context of transcription initiation in pGemU61
Diagrammatic representation of pGemU61 before (A) and after (B) mung bean nuclease treatment and addition of shRNA coding sequence. Blue sequences represent restriction endonuclease recognition sites (or bases derived from recognition sequences). Sequences in bold-type represent RNA polymerase III termination sequences.

3.1.2. Transient transfection of shRNA-GFP

To evaluate the activity pGemU61 we constructed pGemU61-RG. This plasmid encodes an shRNA targeting enhanced green fluorescent protein mRNA (Figure 3.1.2 panel B). The target sequence was based upon a siRNA sequence known to silence hygromycin/EGFP fusion protein expression (Miyagishi and Taira, 2002). Cotransfection of the green fluorescent protein (GFP) expression vector pcDNA3.1Emerald (CMV-EmGFP) and pGemU61-RG in HEK 293-T cells resulted in a ~60% reduction in Emerald GFP (EmGFP) positive cells relative to cells transfected

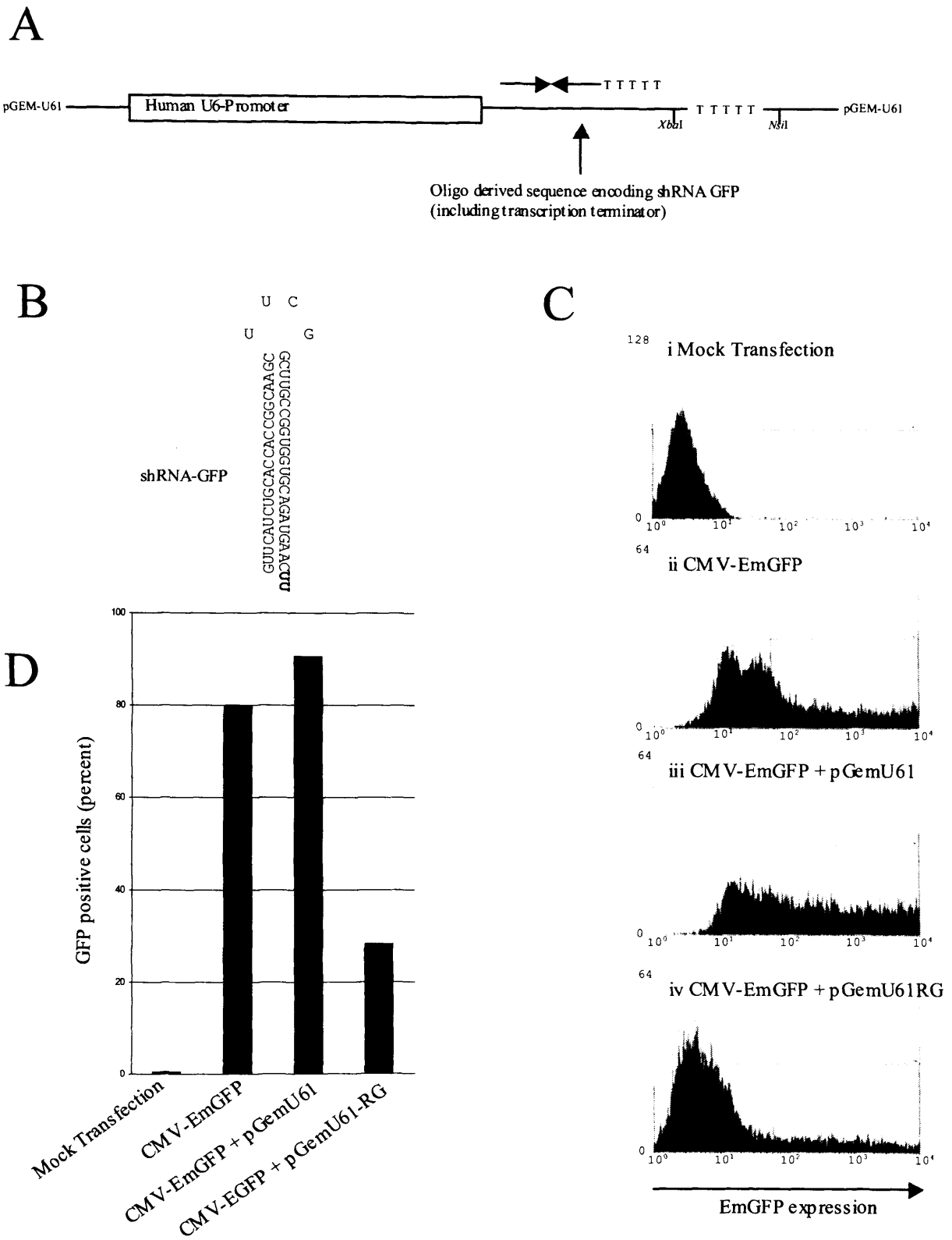


Figure 3.1.2. RNAi with GFP expression in HEK 293-T cells.
A. Schematic of the shRNA expression cassette. B. Probable structure and sequence of shRNA-GFP. C. HEK 293-T cells were transiently transfected with 0.5 µg of an EmGFP expression plasmid (i), 0.5 µg of an EmGFP expression plasmid and 2 µg of a plasmid containing the human U6-promoter (ii) or 0.5 µg of an EmGFP expression plasmid and 2 µg of a plasmid expressing shRNA-GFP (pGemU61-RG) (iv). 48-hours post-transfection GFP expression was assessed, by flow cytometry, and Histograms of FL1-H are shown. D. Graphical representation of the histograms in C indicating the percentage of GFP-positive cells 48-hours post transfection.

with PCDNA3.1Emerald and 'empty' pGemU61 (Figure 3.1.2). EmGFP is a GFP variant with humanised codon usage and 3 amino acid substitutions increasing the intensity of green fluorescence. Crucially, the target sequence of shRNA-GFP is present in both EGFP and EmGFP and the reduction of EmGFP fluorescence in the presence of shRNA-GFP should therefore represent a 'knock down' due to RNAi.

3.2. Lentiviral vector-mediated RNAi

3.2.1. pGemU61-LINKER

HEK 293-T cells can be efficiently transfected. To facilitate the introduction of shRNAs into a greater variety of cells, we generated pGemU61-LINKER (appendix I). Sequences encoding shRNAs can be inserted into pGEMU61-LINKER in an identical fashion to pGemU61. Multiple cloning sites flanking the U6-promoter and inserted gene potentially allow an shRNA expression cassette to be inserted into a variety of viral vectors. Viral vectors should facilitate the introduction of an shRNA cassette into cell lines infected with KSHV.

3.2.2. RNAi in HEK 293-T cells stably expressing dEGFP

To deliver the shRNA cassette we used a lentiviral vector-genome commonly used in our laboratory. This vector genome is encoded by the plasmid pHR'SINcSGW (Demaison *et al.*, 2001), a generous gift from Adrian Thrasher. This SIN lentiviral vector encodes EmGFP driven by an internal spleen focus-forming virus (SFFV) LTR-promoter. To simplify measurements of the 'knock down' in GFP expression, a derivative of pHR'SINcSGW (from here on described as CSGW) expressing DsRed2 was used to deliver the shRNA cassette. This vector encoded by pHR'SINcSRW (from here on described as CSRW) was a gift from Andrew Godfrey. The sequences encoding shRNA-GFP (figure 3.1.2) were inserted into pGemU61-LINKER and the resulting shRNA-GFP cassette was subsequently inserted into CSRW using *Eco* RI. This insertion can occur in 2 possible orientations generating two vector genomes GR1 and GR2 (figure 3.2.1).

To evaluate the potential use of these vectors for gene silencing, we examined the ability of GR1 and GR2 to interfere with GFP expression in the cell line 293-T C1. 293-T C1 cells are a clonal cell line transduced with a lentiviral vector encoding

A

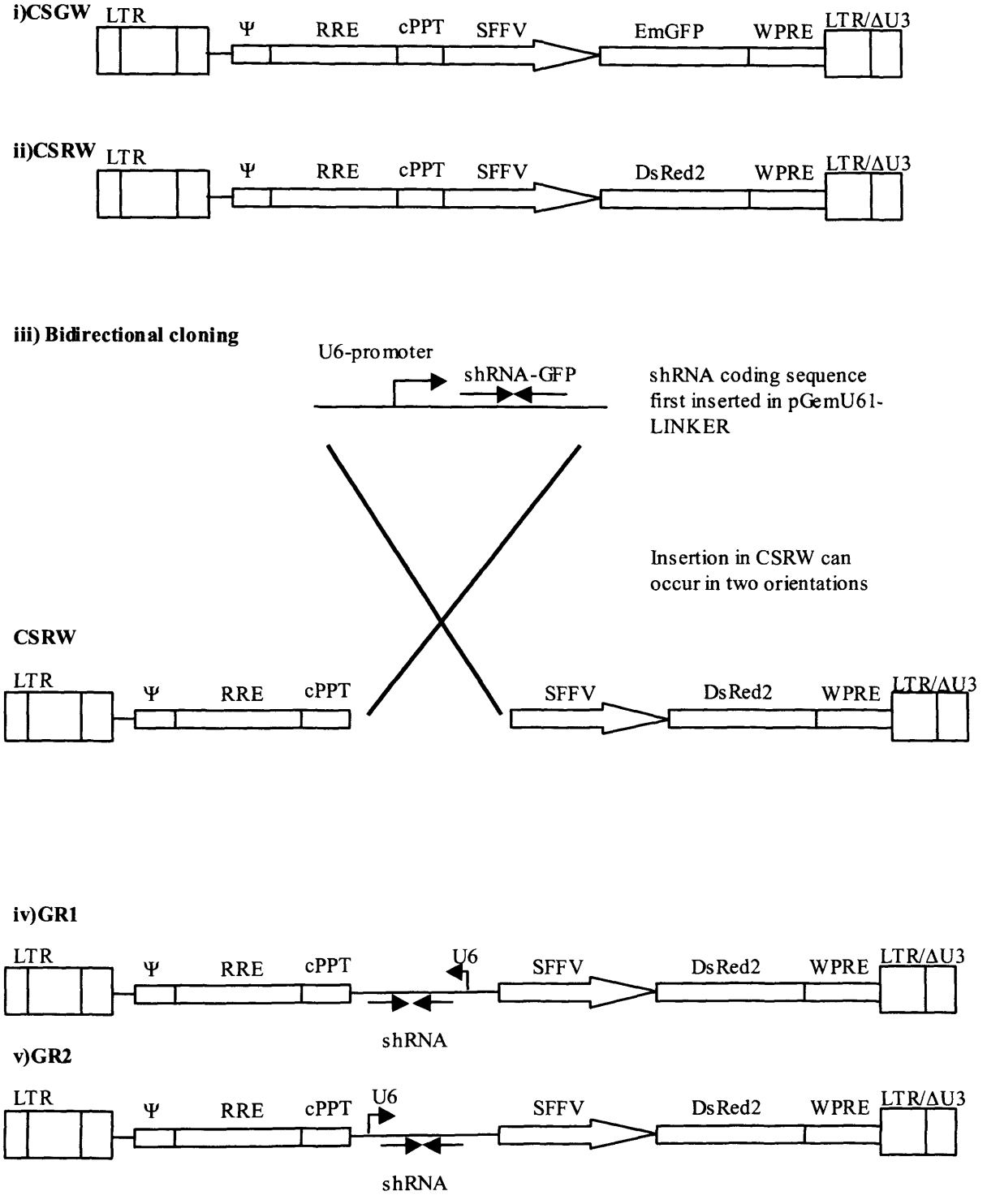


Figure 3.2.1. Lentiviral vector-mediated RNAi with GFP expression is both potent and independent of shRNA expression cassette orientation (continued overleaf).

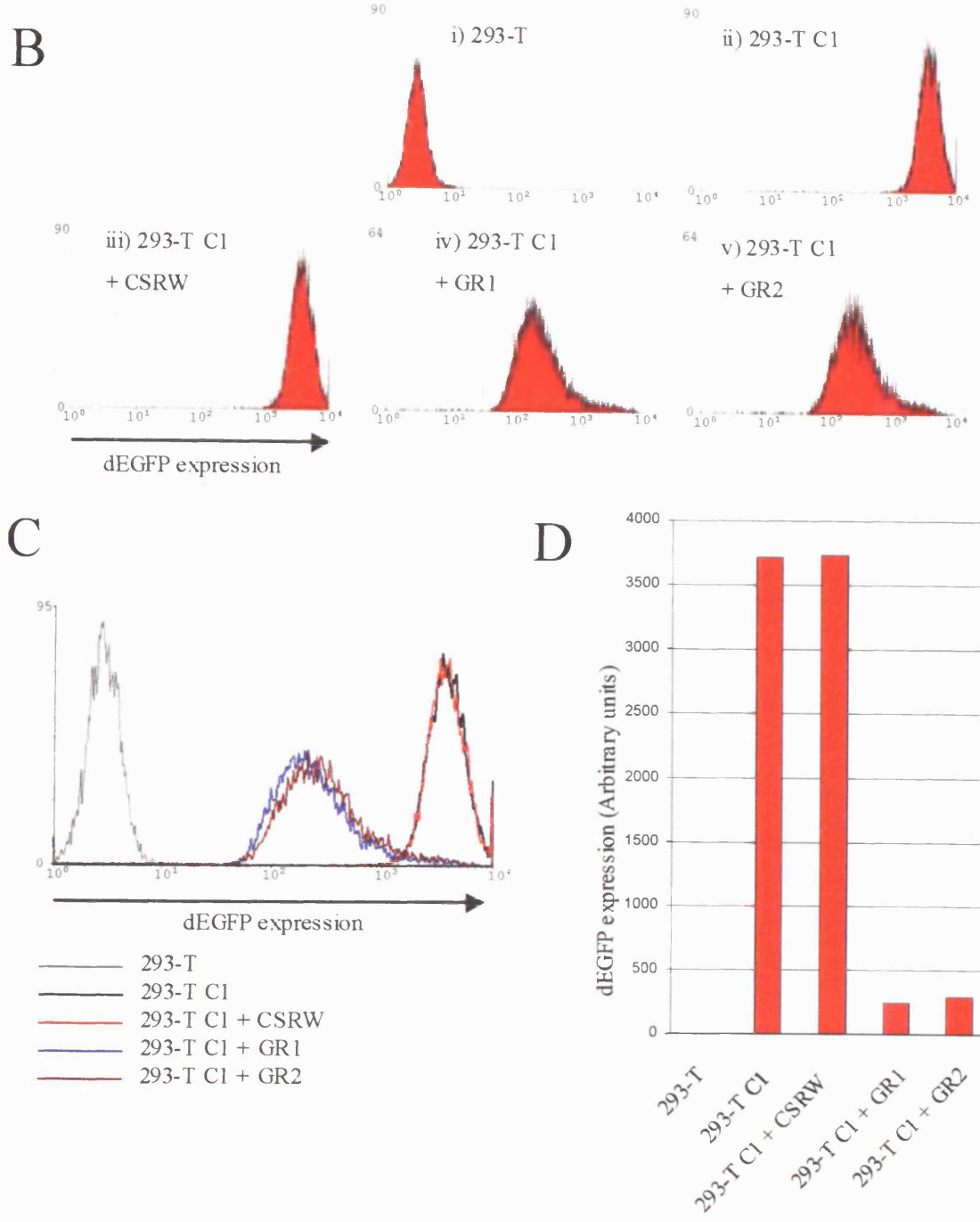


Figure 3.2.1. Lentiviral vector-mediated RNAi with GFP expression is both potent and independent of shRNA expression cassette orientation.
A. Schematic representations of lentiviral vector-genomes of CSGW (i), CSRW (ii), GR1 (iv) and GR2 (v). The insertion of shRNA-GFP into CSRW in two orientations is also summarised (III). The vector genomes contain a Long terminal repeat (LTR), retroviral packaging signal (Ψ), Rev response element (RRE), central poly pyrimidine tract (cPPT), U6-promoter (U6) driven shRNA expression cassette (shRNA), internal spleen focus forming virus LTR (SFFV), coding sequence for DsRed2 (DsRed), coding sequence for Emerald GFP (EmGFP) Woodchuck hepatitis virus polypyrimidine rich element (WPRE) and a self inactivating LTR (LTR/Δ3) B. HEK 293-T C1 cells (ii) were infected with a MOI 10 of CSRW (iii), GR1 (iv) or GR2 (v). 7 days post-infection GFP expression was assessed, by flow cytometry, and Histograms of FL1-H are shown using HEK 293-T cells (i) as an indication of autofluorescence. C. A single histogram plot including all the traces from B. D. GFP expression was quantified for the different samples using the mean fluorescence intensities of the histograms in B.

destabilised GFP (dEGFP), a generous gift from Andrew Godfrey. Destabilised GFP contains the degradation domain of mouse ornithine decarboxylase creating a GFP-variant with a half-life of approximately 2-hours in HEK 293 cells (Li *et al.*, 1998). Transduction of 293-T C1 cells with a MOI 10 of GR1 or GR2 vectors resulted in a ~90% reduction in dEGFP expression whereas cells transduced with CSRW expressed normal levels of dEGFP (Figure 3.2.1). This reduction suggests that 'knockdown' of dEGFP expression and was efficient and independent of shRNA-cassette orientation. It is impossible to completely separate the emission spectra of EmGFP and DsRed2 using our flow cytometers (FACScan and LSR by Becton Dickinson). This could mean the data presented in figure 3.2.1 underestimates the level of 'knock down' mediated by shRNA-GFP.

3.2.3. RNAi using lentiviral vectors conferring resistance to puromycin

The ability of retroviral vectors to integrate into the host-cell genome makes lentiviral vectors potentially suitable for achieving sustained shRNA expression and silencing. To permit selection of cells transduced with shRNA expressing lentiviral vectors, we utilised pHR'SINcSPW (Besnier *et al.*, 2002) a generous gift from Greg Towers. This plasmid encodes a lentiviral vector genome which expresses puromycin acetyl transferase (PAC), an enzyme conferring resistance to the antibiotic puromycin (Vara *et al.*, 1986). We inserted the shRNA-GFP expression cassette into pHR'SINcSPW (from here on described as CSPW) generating the viral vector GP. This vector allows puromycin-selection of transduced cells and the lack of fluorescent transgenes in CSPW vectors makes them particularly amenable to subsequent fluorescent manipulation, of transduced cells, uncomplicated by the emission/absorption spectra of EmGFP/DsRed2.

The benefit of selecting transduced cells is illustrated in figure 3.2.1. To evaluate shRNA expression vectors derived from CSPW, we investigated their efficacy in silencing dEGFP expression in HeLa C1 cells. HeLa C1 cells are a clonal cell line expressing dEGFP generated through transduction of HeLa cells with a lentiviral vector encoding dEGFP (pHR'SINcSDW, a generous gift from A. Godfrey) and subsequent single cell cloning by limiting dilution.

Transduction of HeLa C1 cells with a lentiviral vector input equivalent to a MOI 10 on HEK 293-T cells resulted in a significant reduction in dEGFP expression (figure 3.2.2 panel B). However histogram analysis of dEGFP expression of cells transduced with

GP (figure 3.2.2 panel A) reveals two populations of cells, those similar to normal HeLa cells and those similar to HeLa C1 cells. It seems likely that silencing was effective in transduced cells but that lower infectivity of HeLa cells relative to HEK 293-T cells results in a non-transduced non-silenced population of HeLa C1 cells. If this is correct, selecting transduced cells using puromycin should yield a single silenced population of cells.

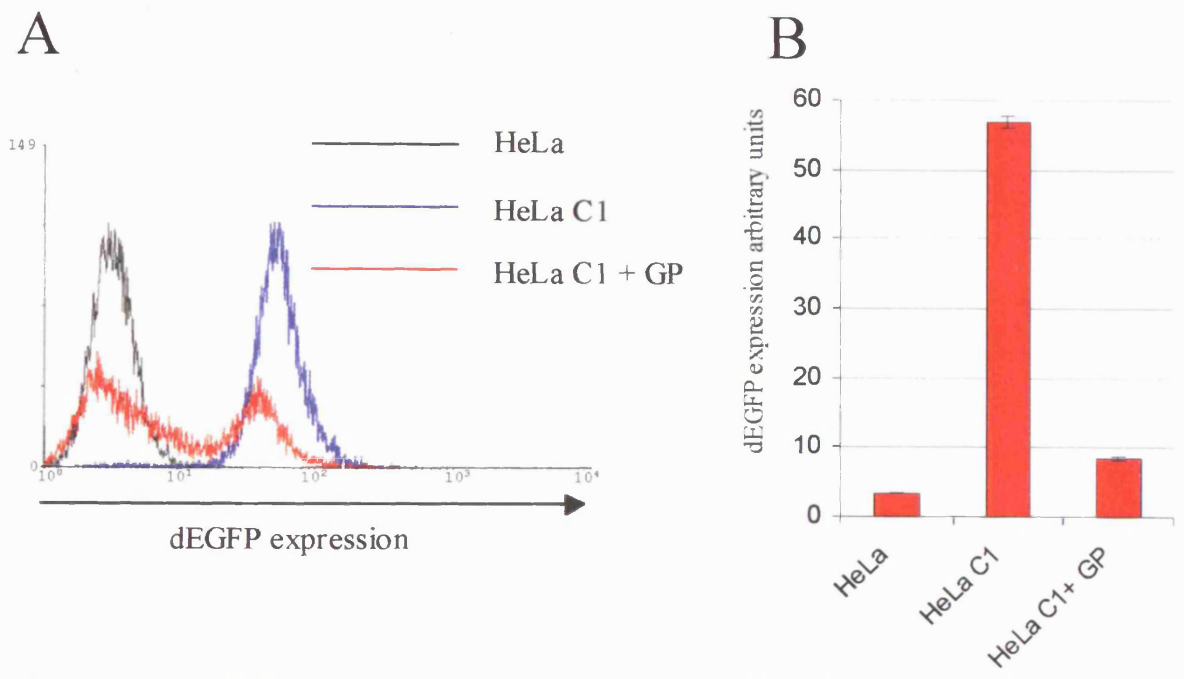


Figure 3.2.2. Transduction of HeLa C1 cells with GP in the absence of puromycin selection.
A HeLa C1 cells (ii) were transduced with a viral vector input equivalent to a MOI 10 on HEK 293-T cells of the lentiviral vector GP. 48-hours post-infection dEGFP expression was assessed, by flow cytometry. Overlaid histograms of HeLa cells (black), HeLa C1 cells (blue) and HeLa C1 cells transduced with GP (Red) are shown. B dEGFP expression was quantified for triplicate samples using the mean fluorescence intensities of the histograms in A. Error bars represent the standard error between triplicate samples.

3.2.4. RNAi in HeLa cells stably expressing dEGFP under puromycin selection

To assess the possibility that non-specific interference with gene expression caused by lentiviral vectors expressing shRNAs might result in the observed decrease in GFP expression, we generated shRNA LacZ (figure 3.2.3 panel A) This shRNA has no complementary target in human cells allowing any difference in silencing between shRNA-GFP and shRNA-LacZ to be attributed to shRNA sequence. In addition, in

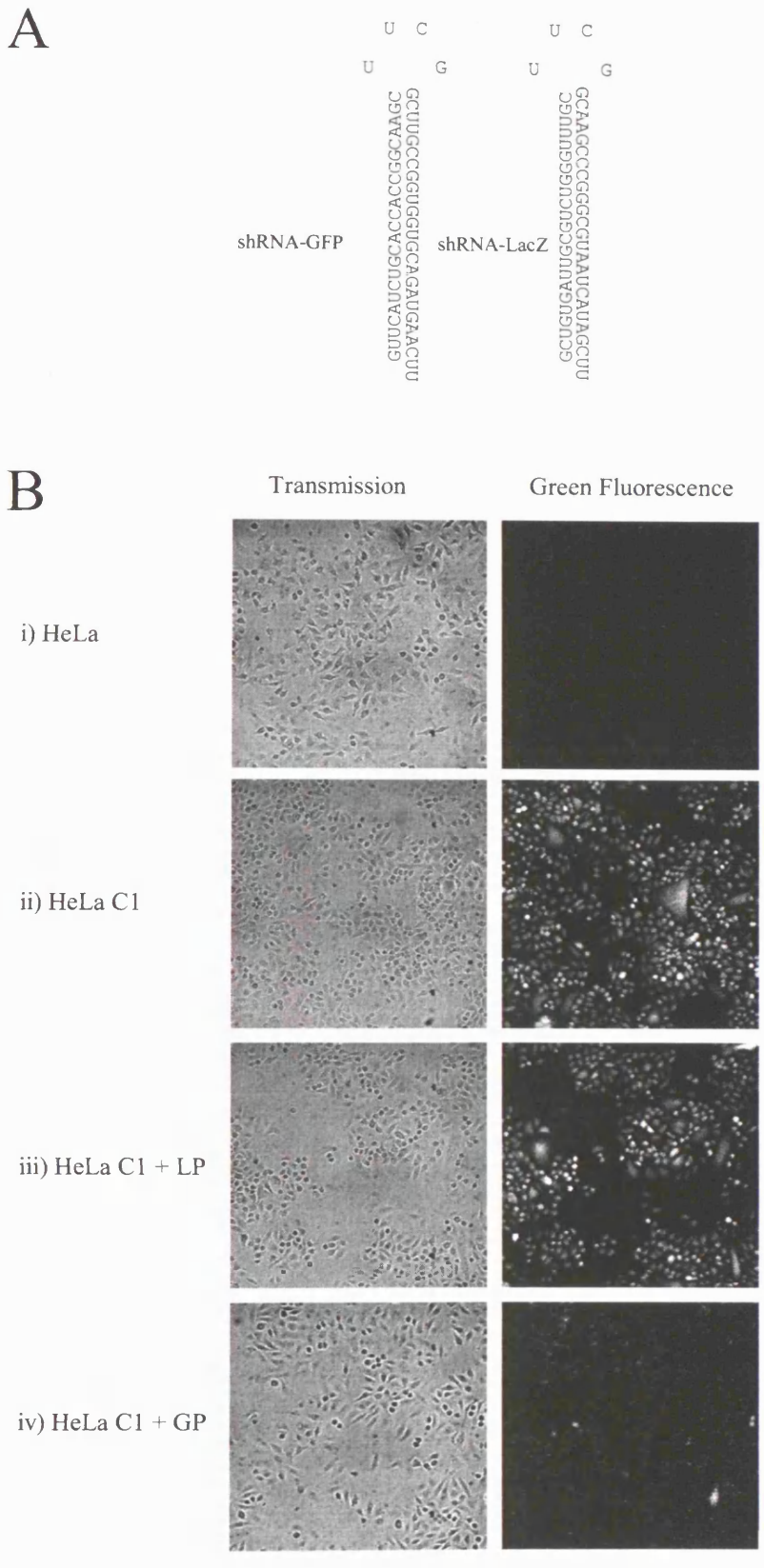


Figure 3.2.3. Lentiviral vector-mediated RNAi with GFP expression in HeLa cells is potent and shRNA sequence specific.

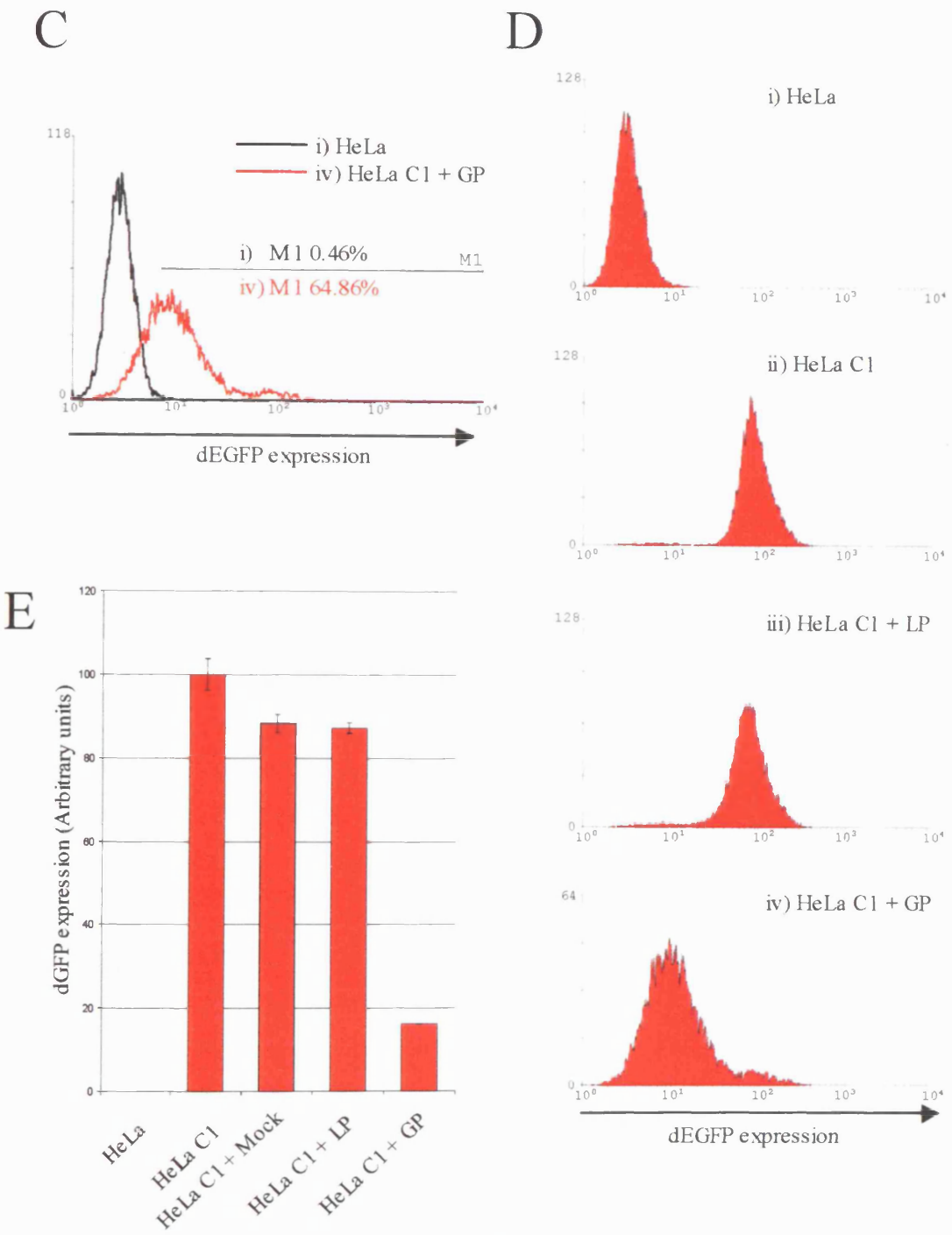


Figure 3.2.3. Lentiviral vector-mediated RNAi with GFP expression in HeLa cells is potent and shRNA sequence specific.
A. Sequence and proposed structure of shRNA-GFP and shRNA-LacZ. B HeLa C1 cells (ii) were transduced with a viral vector input equivalent to a MOI 10 on HEK 293-T cells of LP (iii) or GP (iv) viruses. 48-hours post-infection puromycin selection was applied. 7 days post-infection dEGFP expression was examined using confocal microscopy. Images displaying typical fields of transmission and green fluorescent channels are shown using HeLa cells (i) as an indication of autofluorescence. C As B except 7 days post-infection dEGFP expression was assessed, by flow cytometry. Histograms of HeLa cells (i) and HeLa transduced with GP (iv). The percentage of cells in the M1 population is indicated. D As C, histograms of FL1-H are shown using HeLa cells (i) as an indication of autofluorescence. E. dEGFP expression was quantified for the different samples using the mean fluorescence intensities of the histograms in D. Mock infection, of HeLa C1 cells, with lentiviral vector-free HEK 293-T supernatant is also shown. Error bars represent the standard error between triplicate samples.

light of the dual populations present in HeLa C1 cells transduced with GP virus (figure 3.2.2 A) we assessed the efficacy of GP and LP viral vectors at silencing dEGFP expression in HeLa C1 cells under puromycin selection. 48-hours post transduction puromycin was added to the culture medium. Cells were passaged normally in the presence of puromycin and analysed 7 days postinfection by which time non-transduced HeLa cells in the presence of puromycin were 0% viable (data not shown) indicating that all viable cells passaged in the presence of puromycin had been successfully transduced. Transduction of HeLa C1 cells with a lentiviral vector input equivalent to a MOI 10 on HEK 293-T cells and subsequent puromycin selection resulted in >80% 'knock down' of dEGFP expression (figure 3.2.3). The effect of shRNA-GFP is potent enough that ~35% of GP-transduced HeLa C1 cells fluoresce no brighter than normal HeLa cells (figure 3.2.3 panel C). Mock infections with viral vector-free HEK 293-T supernatant or transduction with shRNA-LacZ expressing vector (LP) resulted in small, similar decreases in dEGFP expression. As predicted, nearly all puromycin-selected shRNA-GFP expressing HeLa C1 cells form a single silenced population suggesting nearly all transduced cells exhibit a silenced phenotype. These data indicate that the 'knockdown' is potent and specific in transduced cells. However, care must be taken to ensure efficient transduction or transduced cells must be selected to achieve silencing in a population of cells.

3.2.5. Successive Transduction and RNAi

To investigate whether shRNA expression was limiting in our system, we repeatedly transduced dEGFP-expressing cells, with different lentiviral vectors, to deliver multiple shRNA expression cassettes per cell. To analyse the affect of serial transduction of shRNA expressing lentiviral vectors on dEGFP expression, HEK 293-T C1 cells were transduced with a MOI 10 of LP or GP viral vectors on 3 occasions at 4-day intervals. The results of this investigation are shown in figure 3.2.4. The majority of dEGFP expression (~90%) is removed following initial transduction with the lentiviral vector GP. Subsequent transduction causes minimal further reduction in dEGFP expression. Interestingly, successive transduction with LP also reduces dEGFP expression. Histogram analysis of dEGFP expression suggests the decreased mean fluorescence intensities result from a reduction in dEGFP expression in the majority of cells, rather than transduction of cells not transduced in previous infections (data not shown). The observation that successive transduction by GP and LP lentiviral vectors decreases

dEGFP expression independent of shRNA sequence, suggests this reduction is not due to RNAi but some other non-specific process.

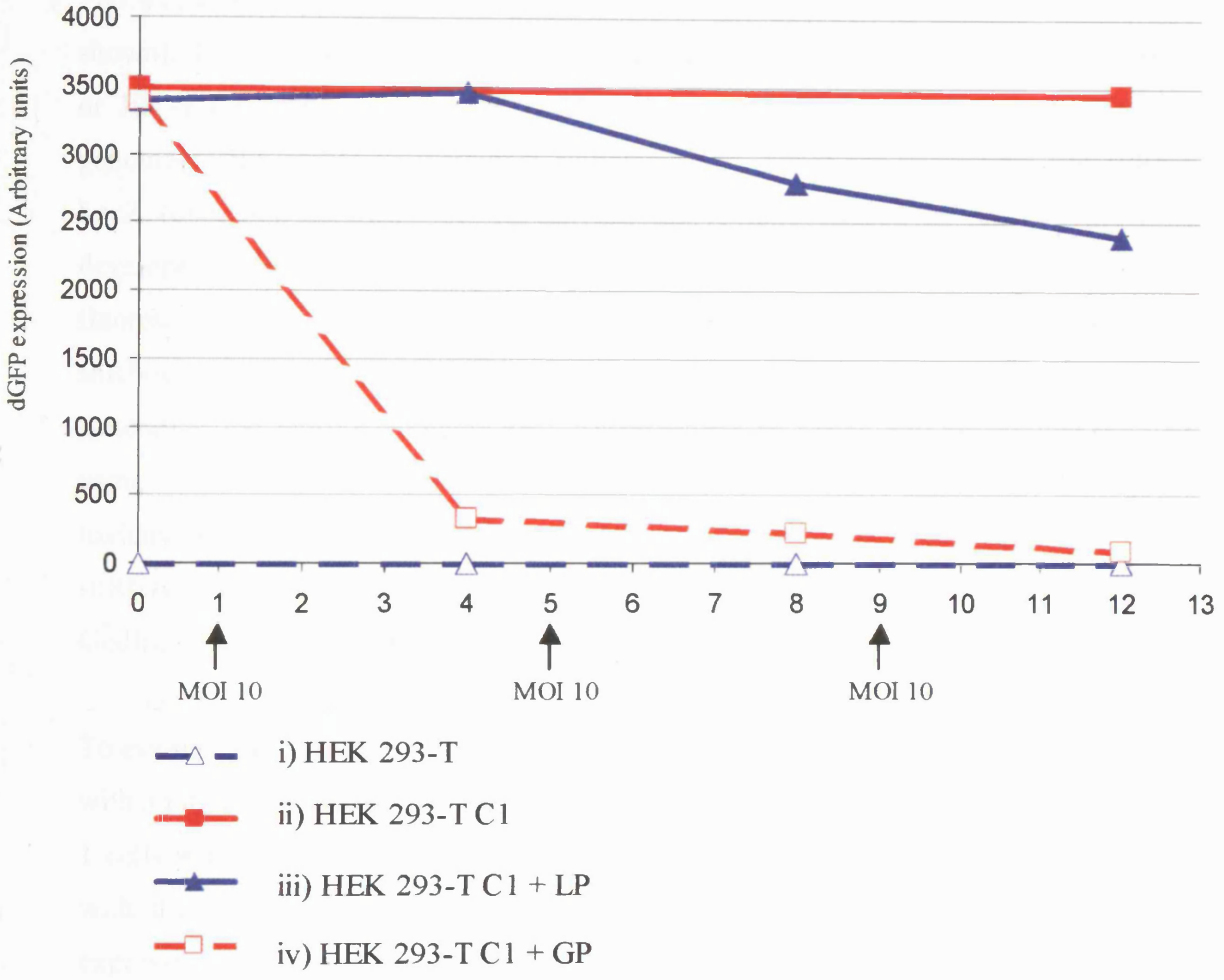


Figure 3.2.4. Successive rounds of lentiviral vector infection do not greatly enhance gene silencing.
293-T C1 cells (ii) were infected with a MOI 10 of GP (iii) or LP (iv) viruses on days 1, 5 and 9. On days 0, 4, 8 and 12 days post-infection GFP expression was assessed, by flow cytometry using HEK 293-T cells (i) as an indication of autofluorescence. GFP expression was quantified for the different samples using mean fluorescence intensities and these are displayed plotted against the time in days (x-axis).

3.2.5. Lentiviral vector-mediated RNAi in PEL cells

We wanted to develop an RNAi vector system that could be used to 'knock down' KSHV open reading frames (ORFs) to allow investigation of host-virus relationships. To evaluate the potential of this system in effecting RNAi in cells latently infected with KSHV we examined the activity of GP and LP vectors in the PEL cell line JSC-1 C1.

This dEGFP expressing clonal cell line was generated using pHR'SINcSDW-derived lentiviral vector in identical fashion to HeLa C1 cells. Surprisingly, shRNA-LacZ is extremely toxic in PEL-derived JSC-1 cells. Transduction of JSC-1, JSC-1 C1 or PEL derived BC-3 cells with an input equivalent to a MOI 5 on HEK 293-T cells results in >80% cell mortality 48-hours postinfection (adjudged by trypan blue exclusion, data not shown). Despite repeated attempts, we were unable to derive puromycin resistant JSC-1 or JSC-1 C1 cultures using LP. BLAST searches of the human, KSHV or EBV genomes were unable to identify a complementary target for either strand of shRNA LacZ (data not shown). To circumvent the toxicity caused by shRNA LacZ we developed shRNA-DsRed (figure 3.2.5 panel A). This shRNA is targeted to the fluorescent protein DsRed2 and has no complementary target in human cells. This shRNA was developed in collaboration with Andrew Godfrey and inserted into CSPW generating the lentiviral vector RP. Transduction of JSC-1 and BC-3 cells with RP using an input equivalent to a MOI 10 on HEK 293-T cells showed no obvious signs of toxicity (data not shown). Although RP is used solely as a control in this thesis, shRNA-DsRed is an active shRNA effecting efficient knockdown of DsRed (Andrew Godfrey personal communication).

To evaluate the silencing activity of GP and RP vectors, JSC-1 C1 cells were transduced with an input equivalent to a MOI 10 on HEK 293-T cells. 48-hours postinfection JSC-1 cells were fixed and dEGFP expression analysed by flow cytometry. Transduction with the shRNA-GFP expressing vector GP resulted in >80% decrease in dEGFP expression (figure 3.2.5 panel C) relative to mock and RP transduced cells. The interference with dEGFP expression was specific to GP transduced cells suggesting the 'knock down' was due to RNAi with gene expression.

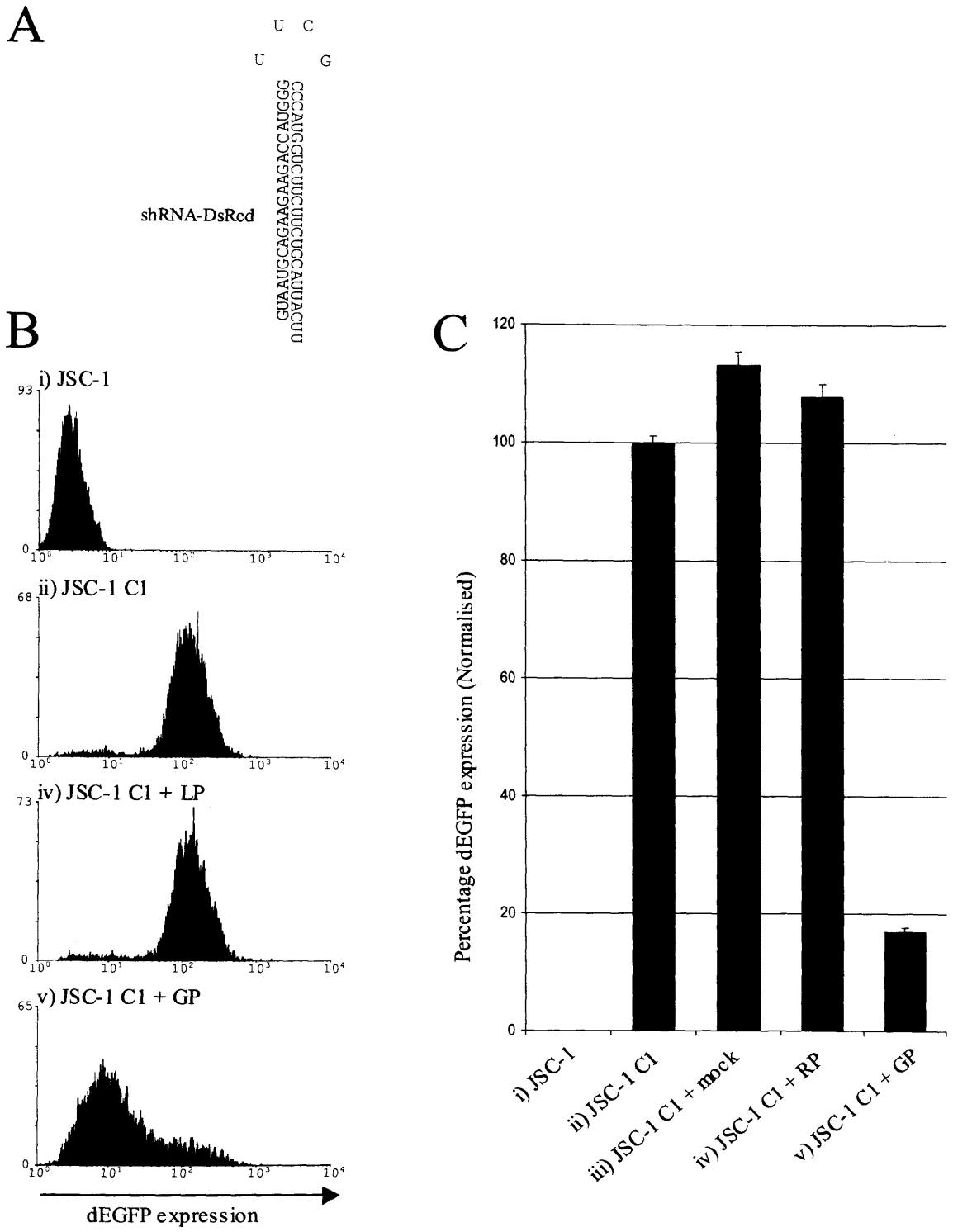


Figure 3.2.5. Lentiviral vector-mediated RNAi with GFP expression in KSHV positive JSC-1 cells.

A. A diagrammatic representation of shRNA-DsRed. B. JSC-1 C1 cells (ii) were infected with a viral vector input equivalent to a MOI 10 on HEK 293-T cells of LP (iv) or GP (v) viruses. 48 h post-infection GFP expression was assessed, by flow cytometry, and Histograms of FL1-H are shown using JSC-1 cells (i) as an indication of autofluorescence. C. GFP expression was quantified for the different samples using the mean fluorescence intensities of the histograms in A. A mock infection using HEK-293-T supernatant is also included (histogram not shown). Error bars represent the standard error between triplicate samples.

Discussion I

3.3. Lentiviral vector-mediated RNAi

The aim of this work was to produce an, RNA polymerase III promoter driven, shRNA expression system capable of being delivered using lentiviral vectors and mediating RNAi. In all cell lines tested (HEK 293-T, HeLa and JSC-1) we were able to demonstrate the reduction of GFP expression mediated by shRNA-GFP. This reduction is specific to shRNA-GFP and is largely unaffected by shRNA-LacZ, shRNA-DsRed, or lentiviral vector transduction. The shRNA expression cassette is functional in either orientation within the lentiviral vector backbone. This suggests the proximity of the U6-promoter to the internal SFFV LTR promoter does not result in significant promoter interference and also facilitates simple cloning of the shRNA expression cassettes. These modified lentiviral vectors are capable of reducing their target gene expression by ~80-90% in multiple cell lines. These vectors are also able to 'knock down' dEGFP expression in KSHV infected PEL cell lines indicating their potential utility in the 'knock down' of KSHV open reading frames.

The magnitude of 'knock down' and the shRNA specificity of this response suggest that RNAi is the mechanism by which shRNA-GFP reduces EmGFP/dEGFP expression. However, some non-specific effects were observed. The shRNA-LacZ hairpin exhibits significant toxicity in PEL cell lines. We were unable to identify a complementary target for either strand of shRNA-LacZ and it is likely that shRNA-LacZ is either engaged in off-target interference with essential gene expression or is a potent activator of the interferon response. We believe it is the former because HeLa cells are capable of mounting an interferon response following transduction with lentiviral vectors expressing shRNAs (Bridge *et al.*, 2003). However, shRNA LacZ showed no obvious signs of toxicity in HeLa cells. Alternatively, the increased toxicity in PEL cell lines could represent the differential downstream response of PEL cell lines and HeLa cells to dsRNA. The authentic context of U6 transcription initiation from pGemU61-LINKER, should however, make these shRNAs minimal inducers of an interferon response (Pebernard and Iggo, 2004). No other shRNA used in our laboratory has shown similar toxicity in JSC-1 cells.

Using lentiviral vectors to express shRNAs potentially allows sustained gene silencing. Puromycin selection of shRNA-GFP transduced HeLa C1 cells generates a single

population of cells with respect to green fluorescence intensity (Figure 3.2.3 D), whereas non-selected cells have two distinct populations (figure 3.2.2 A). Assuming a single vector genome integration confers puromycin resistance, this suggests that a single vector genome integration is sufficient to mediate potent RNAi with dEGFP expression. The concentration dependence of off-target silencing and interferon induction suggest that minimising the viral-vector input should minimise non-specific effects whilst maintaining effective silencing.

There are currently many commercially available shRNA expression systems. However, when this work commenced lentiviral vector-mediated RNAi was an unpublished strategy, leading us to develop our own system. Fortuitously the authentic context of transcription initiation and >19 bp shRNA duplexes may enhance the specificity and magnitude of silencing achieved by our vectors (Pebernard and Iggo, 2004; Siolas *et al.*, 2005). Despite the multitude of alternative vector systems, the SFFV promoter is highly active in cells of a haematopoietic lineage (Demaison *et al.*, 2002) and CSGW derivatives can be produced to relatively high titres, making this system useful in cells such as stem cells and peripheral blood mononucleocytes which are otherwise difficult to manipulate. In the absence of a consensus concerning the best strategy for shRNA expression, these vectors continue to provide a useful tool for achieving sustained gene silencing (Ylinen *et al.*, 2005).

Chapter 4

Results: RNAi with KSHV ORF50 Expression

4.1. Screening shRNAs

4.1.1. Screening shRNAs targeting KSHV ORF50

The previous chapter described potent interference with exogenous dEGFP expression in JSC-1 cells harbouring latent KSHV. To evaluate the utility of our lentiviral shRNA expression system, in interfering with viral gene expression, we targeted KSHV ORF50. KSHV ORF50 is necessary and sufficient to initiate the KSHV lytic replication cycle (Lukac *et al.*, 1998) allowing the affect of shRNAs targeting ORF50 to be assessed in terms of their potency in reducing ORF50 expression and their affect on KSHV lytic replication. Reducing the expression of a well-characterised viral protein provides proof of principle that viral gene expression can be reduced and the phenotypic consequences observed using our lentiviral shRNA expression system.

No siRNAs or shRNAs targeting KSHV ORF50 have been described so 3 shRNAs were tested empirically for their activity in reducing KSHV RTA protein expression. The three shRNAs were targeted to different regions of ORF50 mRNA (indicated in figure 4.1.1 A/B) including the splice junction (shRNA-50SJ), the middle of the transcript (shRNA-50M) and one nearer the 3' end of the transcript (shRNA-50E). These shRNAs are expressed from a CSPW background (section 3.2.3) and are encoded by the lentiviral vectors SJP, MP and EP respectively. JSC-1 cells were transduced with an input equivalent to a MOI 10 on HEK 293-T cells of SJP, MP, EP, RP and CSPW. Transduced cells were puromycin selected and 8-days postinfection the lytic cycle was induced through TPA treatment. 24-hours later cells were fixed, permeabilised and stained for KSHV RTA expression using a monoclonal antibody specific for KSHV RTA (Okuno *et al.*, 2002), a generous gift from Keiji Ueda. Analysis of RTA expression by flow cytometry indicated that shRNA-DsRed (encoded by RP) and shRNA-50SJ (encoded by SJP) had little affect on KSHV RTA expression whereas shRNA-50M (encoded by MP) and shRNA-50E (encoded by EP) prevented RTA expression in approximately 70% or 80% of TPA induced JSC-1 cells respectively (figure 4.1.1.C).

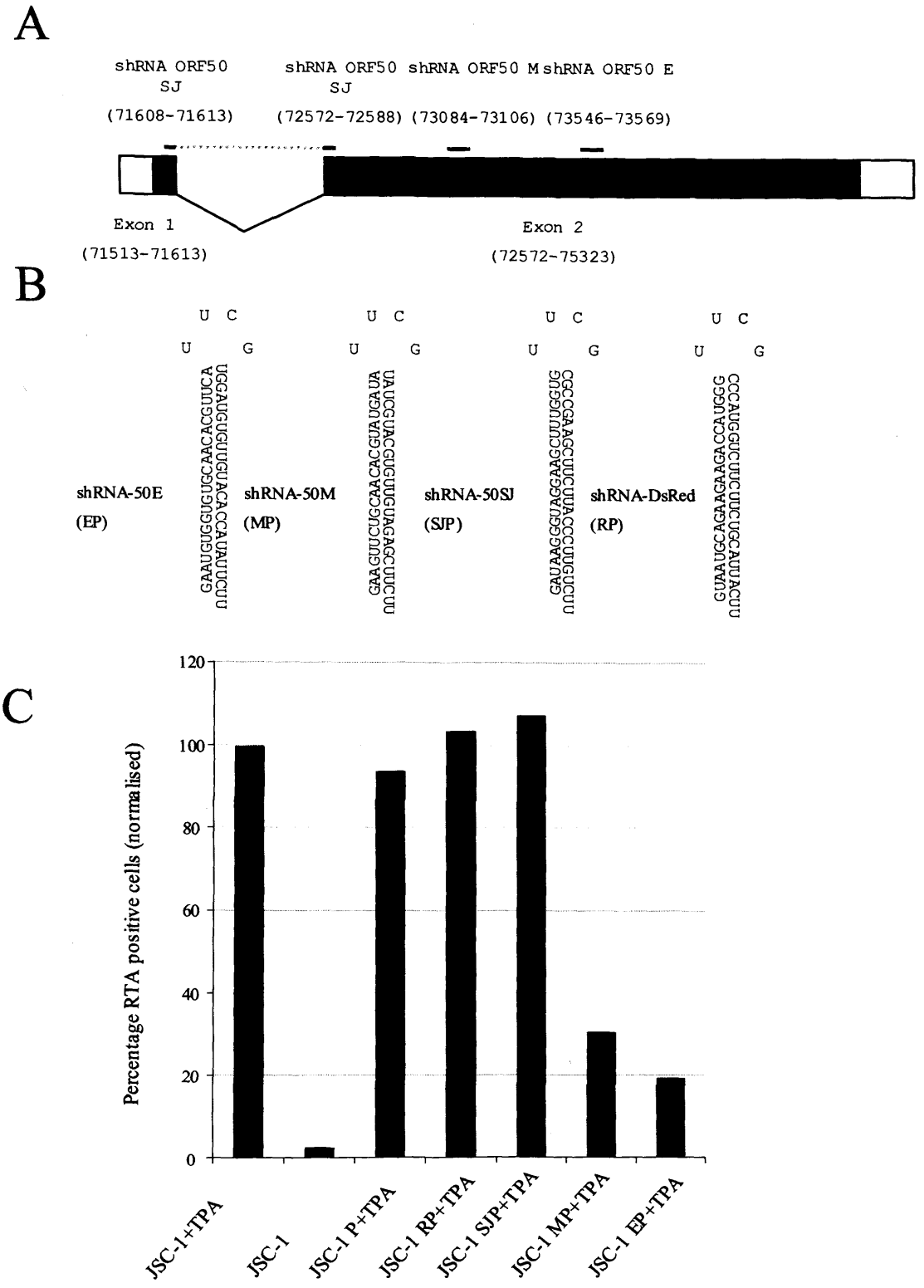


Figure 4.1.1. Screening shRNAs targeting KSHV ORF50 (RTA).
A. Diagram showing the position of sequences targeted by shRNAs in ORF50 mRNA. The numbering corresponds to KSHV genomic sequence (gi: 18845965). B. Probable structure and sequence of shRNAs targeting KSHV ORF50 (50SJ, 50M and 50E) and DsRed. C. JSC-1 cells were infected with lentiviral vectors CSPW (P), RP, SJP, MP and EP with an input equivalent to a MOI 10 on HEK 293-T cells. 2 days post-infection a pure population of transduced cells was generated through puromycin selection. 8-days post-infection transduced and not transduced JSC-1 cells were treated with TPA for 24 hours before being fixed and stained for RTA expression. Using flow cytometry the percentage of RTA positive cells was calculated then normalised to the percentage of RTA positive TPA induced JSC-1 cells (30.29%). Non-induced JSC-1 cells were also fixed and stained to give an indication of spontaneous lytic replication.

4.2. Characterising shRNA-50E

4.2.1. shRNA-50E in distinct cell lines

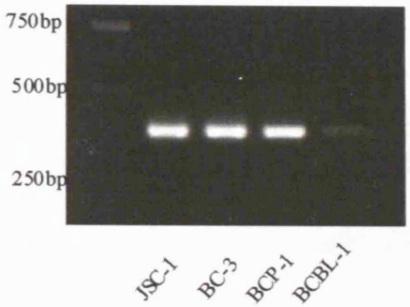
Only 1 complete KSHV genome sequence exists (Accession: NC_003409) generated from KSHV DNA cloned from the PEL cell line BC-1 (Russo *et al.*, 1996). The sequence variation of KSHV ORF50 has not been the subject of direct investigation. To confirm the presence of the most potent shRNA target sequence (shRNA-50E) in distinct cell lines harbouring KSHV of different subtypes, the target region was PCR-amplified, using oligonucleotides 28 and 29, from different cell lines and sequenced. The cell lines JSC-1, BC-3, BCP-1 and BCBL-1 were used for this analysis and are infected with KSHV subtypes C3/P, C3/P, A1/P and A3/P respectively (Zong *et al.*, 2002). An alignment of the shRNA-50E target region is displayed in figure 4.1.2 B indicating the invariant nature of the target sequence in all cell lines tested. To date we have no information regarding the target sequence in KSHV from the less common clades B and D.

Although fewer KSHV positive BC-3 cells enter the lytic cycle following TPA-treatment, analysis of shRNA-50E in JSC-1 and BC-3 cell lines indicates that approximately 80% of cells which would normally express RTA do not express detectable levels 24-hours postinduction (Figure 4.1.2). Rather than reducing the RTA protein levels in all RTA-positive cells, shRNA-50E blocks RTA expression in the majority of cells. The minority of cells expressing RTA, in the presence of shRNA-50E, do so at levels comparable to untreated cells (adjudged by the mean fluorescence intensity of RTA positive cells). The ability of KSHV RTA to auto-activate the KSHV ORF50 promoter (Deng *et al.*, 2000) led us to consider the possibility that shRNA-50E delayed the positive-feedback effect of RTA, thereby delaying RTA expression. To examine this possibility, RTA expression was analysed over a 72-hour period.

4.2.2. Analysis of shRNA-50E over 72-hours in JSC-1 cells

JSC-1 cells were transduced with an input equivalent to a MOI 10 on HEK 293-T cells of SJP and EP. Transduced cells were puromycin selected and 8 days postinfection the lytic cycle was induced by TPA treatment for 1-hour, after which the cells were washed 3 times in culture medium, and incubated for 3, 6, 12, 24, 48 and 72-hours. At each time point $3x10^6$ cells were fixed and stored at 4°C for subsequent analysis. After 72-

A



B

```
                                    U
                GAAUGUGGUGUGCAACACGUUCA     C
                UUCUACUAUGUUGUGACACCAUAUUCUU
                                    G
GCCGCGTCACAGAATATGGTGTACAACACATCCACCGCGCCCTTAAAAC BCP1
GCCGCGTCACAGAATATGGTGTACAACACATCCACCGCGCCCTTAAAAC JSC1
GCCGCGTCACAGAATATGGTGTACAACACATCCACCGCGCCCTTAAAAC BCBL1
GCCGCGTCACAGAATATGGTGTACAACACATCCACCGCGCCCTTAAAAC BC3
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C

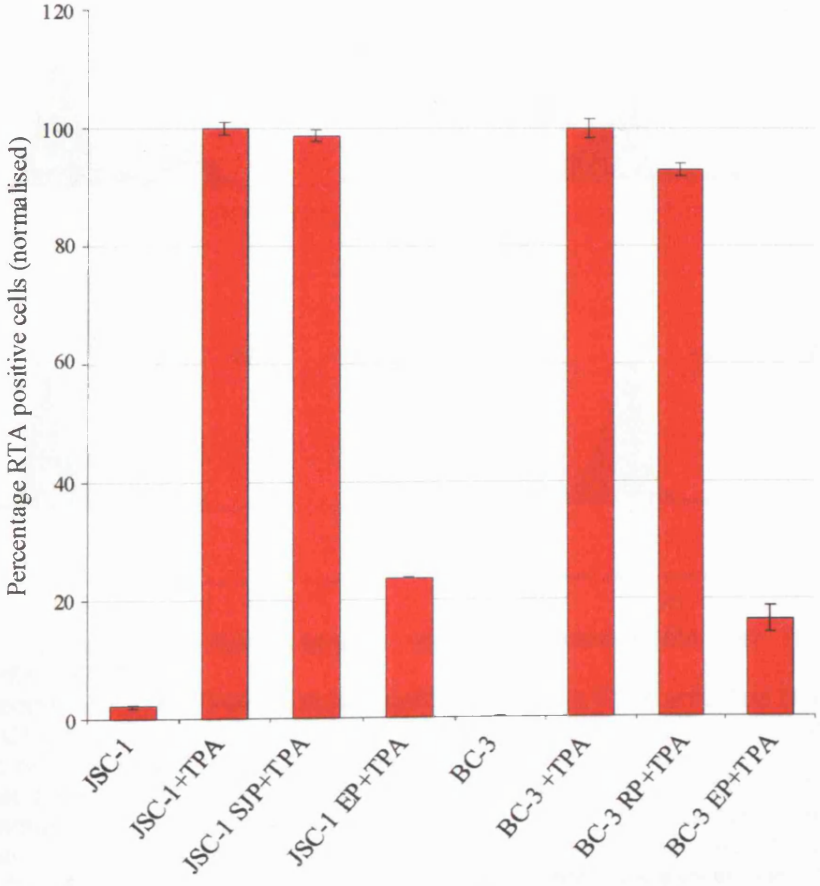


Figure 4.1.2. Reproducible lentiviral vector-mediated RNAi with RTA expression in distinct PEL cell lines (continued overleaf).

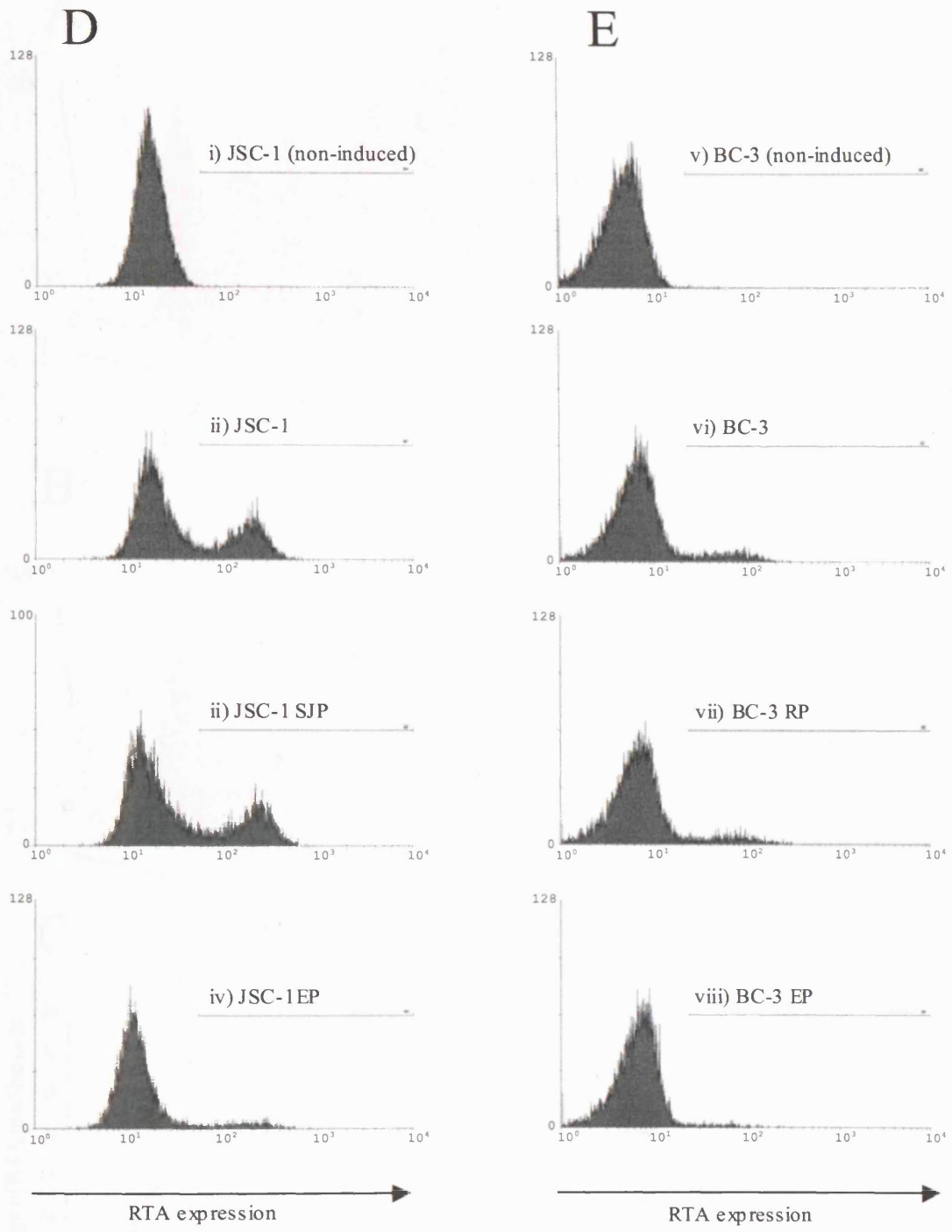


Figure 4.1.2. Reproducible lentiviral vector-mediated RNAi with RTA expression in distinct PEL cell lines.

A. The region of KSHV DNA encoding shRNA-50E was PCR amplified from 4 PEL cell lines and the PCR product visualised by ethidium bromide stained agarose gel electrophoresis. B. A schematic representation of cloned and sequenced PCR products from A. * Indicates identical bases in all the PEL cell lines sampled. The shRNA target region is highlighted in red with the complementary region of shRNA-50E adjacent to it. C. A graphical representation of RTA expression in JSC-1 and BC-3 cells. Transduced and not transduced PEL cells were induced with TPA for 24-hours before being fixed and stained for RTA expression. The percentage of RTA positive cells was generated as in figure 4.1.1 and normalised to the percentage of RTA positive TPA treated PEL cells (JSC-1 32.67%, BC-3 10.28%). Transduced JSC-1 and BC-3 cells were generated by the same method outlined in figure 4.1.1. Error bars represent the standard error between triplicate experiments. D. Typical histograms of RTA expression using JSC-1 cells and transduced JSC-1 cells generated in figure 4.1.1. E. Typical histograms of RTA expression in transduced and not transduced BC-3 cells.

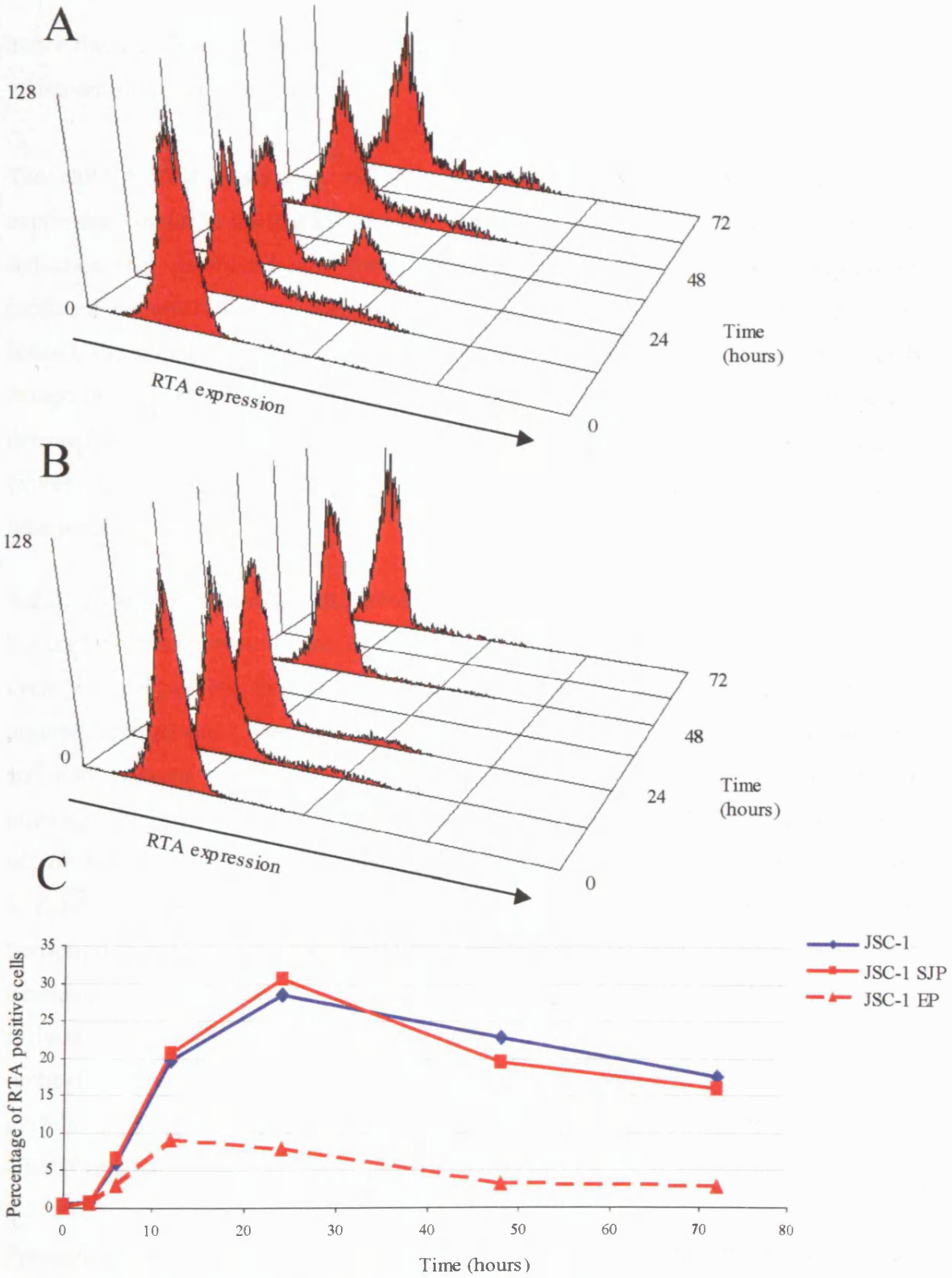


Figure 4.2.2. Analysis of RTA expression in shRNA ORF50 expressing cells.
A. JSC-1 cells transduced with the control lentiviral vector expressing shRNA-50SJ were treated with TPA to induce KSHV lytic replication. Cells were harvested at various time points, fixed and stained for RTA expression and histograms of RTA expression are shown. B. JSC-1 cells transduced with a lentiviral vector expressing shRNA-50E were treated as in A and histograms of RTA expression are shown. C. The percentage of RTA positive cells analysed by flow cytometry was calculated at different time points for not transduced JSC-1 cells and cells transduced with shRNA expressing lentiviral vectors and is represented graphically. All JSC-1 cells were transduced and puromycin selected as described in figure 4.1.1.

hours fixed cells were permeabilised and stained for RTA expression, the results of which are shown in figure 4.2.2.

The shRNA-50SJ expressing JSC-1 cells display a profile of TPA-induced RTA expression similar to normal JSC-1 cells, with a peak of RTA expression 24-hours post-induction that decreased over time. It is clear that RNAi with RTA expression, mediated by shRNA-50E, does not result in a delay in maximal RTA expression. Indeed, the profile of RTA expression is similar to normal TPA-induced JSC-1 cells except that far fewer cells express RTA. Interestingly, maximal RTA expression is detected earlier in cells expressing shRNA-50E than in normal or control shRNA expressing cells. The apparent block in lytic induction is closer to 50% at these earlier time points.

4.2.3. Analysis of early lytic proteins

To confirm that prevention of RTA expression results in a block to the KSHV lytic cycle we analysed the expression of KSHV ORFK8 and ORF59. KSHV ORFK8 is a multiply spliced early gene encoding K-bZIP/RAP/K8α and potentially encoding K8β, and K8γ proteins (Lin *et al.*, 1999). The K-bZIP protein acts as a transcriptional repressor (Izumiya *et al.*, 2003) and is also involved in KSHV DNA replication (Lin *et al.*, 2003a). Although K8 exons are included in immediate early transcripts KIE1, 2 and 3, K-bZIP is only expressed from transcripts driven by the K8 promoter. Lytic cycle transcription from the K8 promoter is sensitive to cycloheximide but insensitive to PAA treatment (Lin *et al.*, 1999), designating K8 as a true early gene. KSHV ORF59 is an early-late gene encoding processivity factor 8 (PF-8), which is involved in KSHV DNA replication (Chan *et al.*, 1998; 2000). Lytic cycle transcription of ORF59 is sensitive to cycloheximide and partially sensitive to PAA treatment (Chan *et al.*, 1998), designating ORF59 as a partial-late (γ1) gene.

Preventing RTA expression should prevent all early and late gene expression (Xu *et al.*, 2005). The analysis of, the early genes, K-bZIP and PF-8 expression should indicate whether the KSHV lytic cycle has been faithfully blocked by shRNA-50E. To investigate this hypothesis we examined K-bZIP and PF-8 expression in JSC-1 cells.

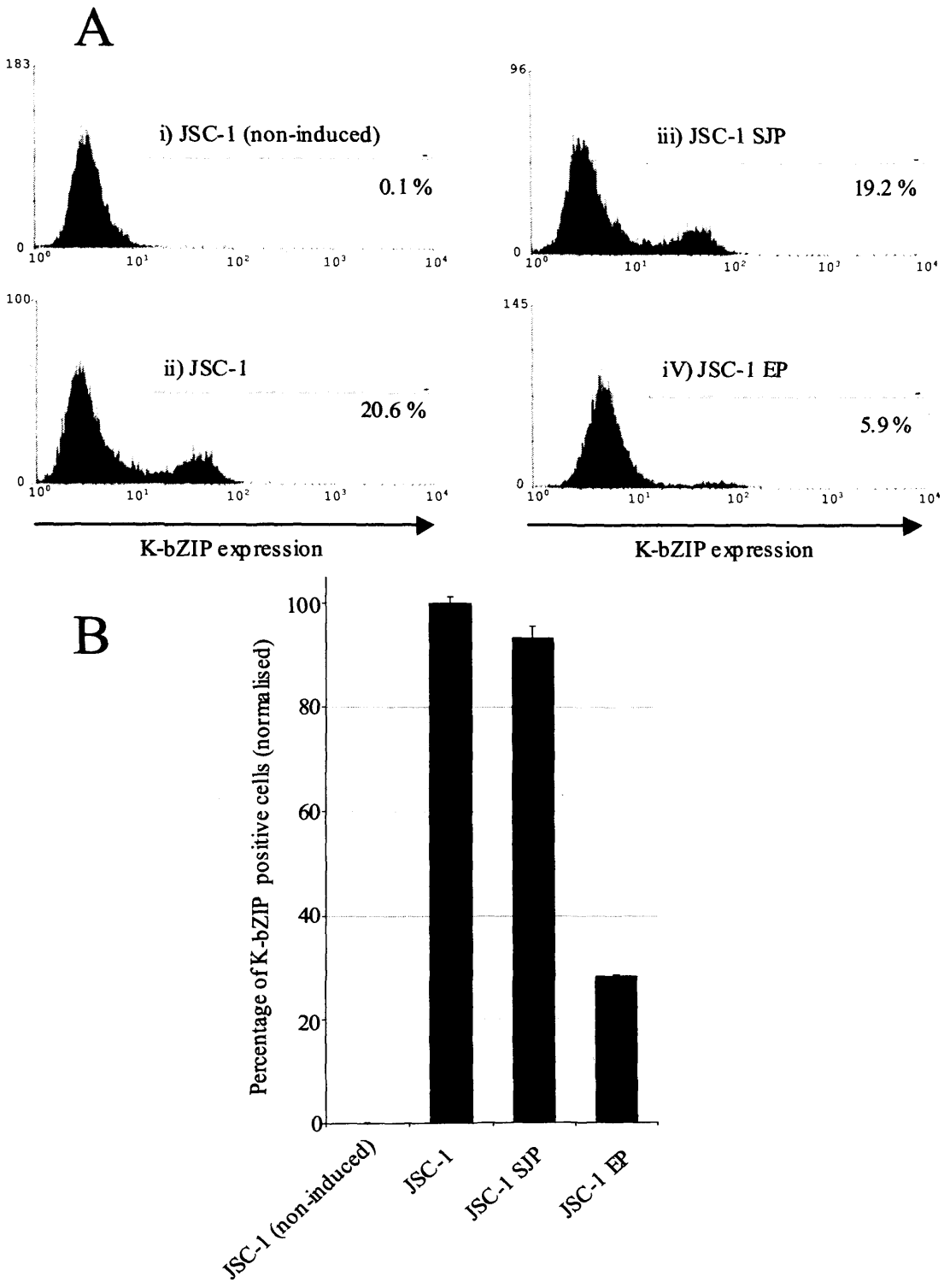
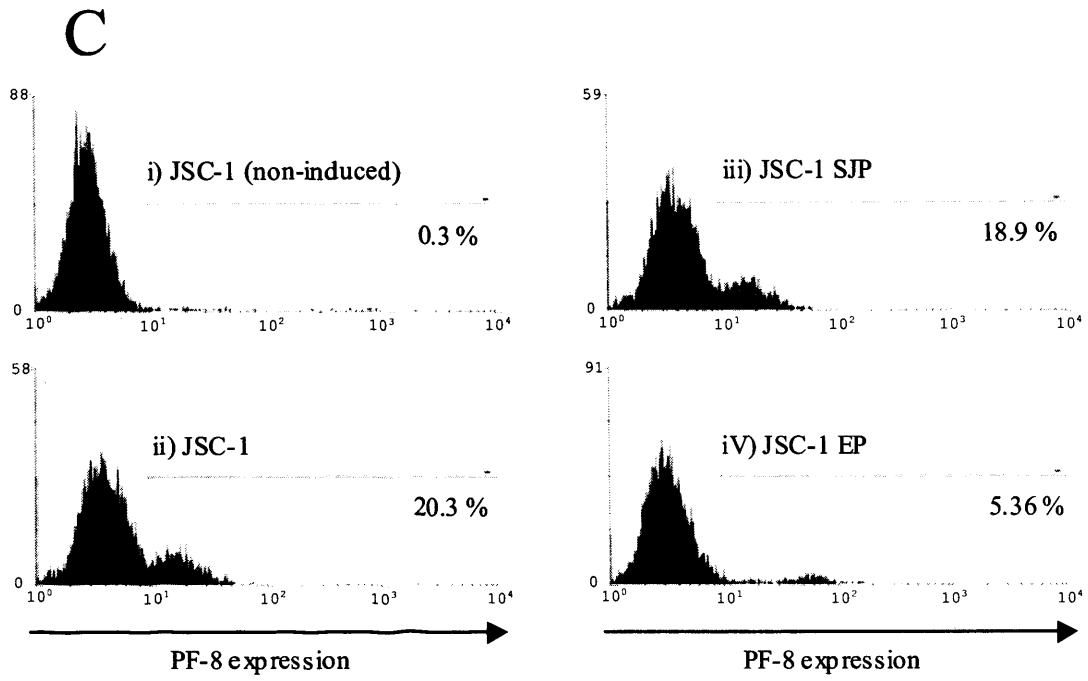


Figure 4.2.3. Expression of lytic proteins downstream of RTA is reduced by shRNA 50E (continued overleaf).



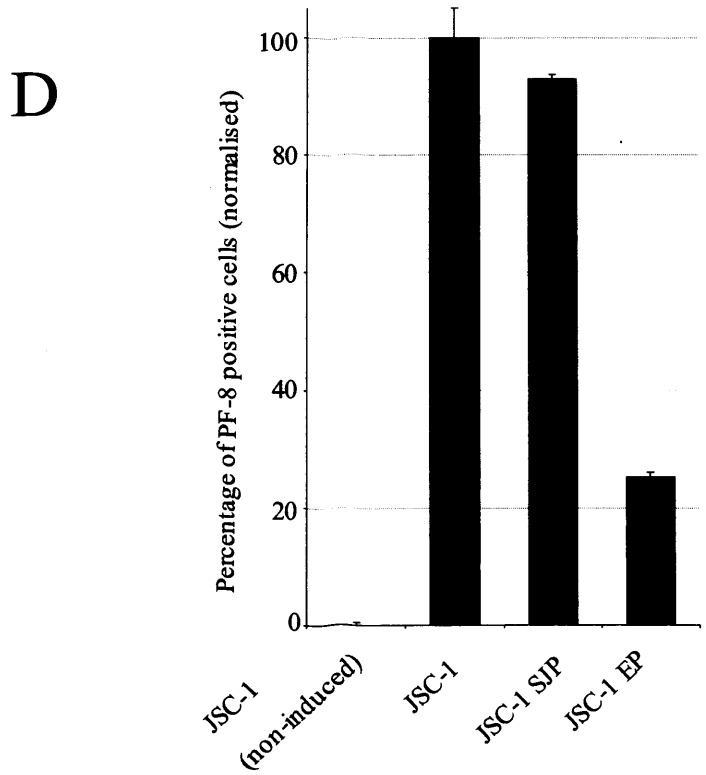
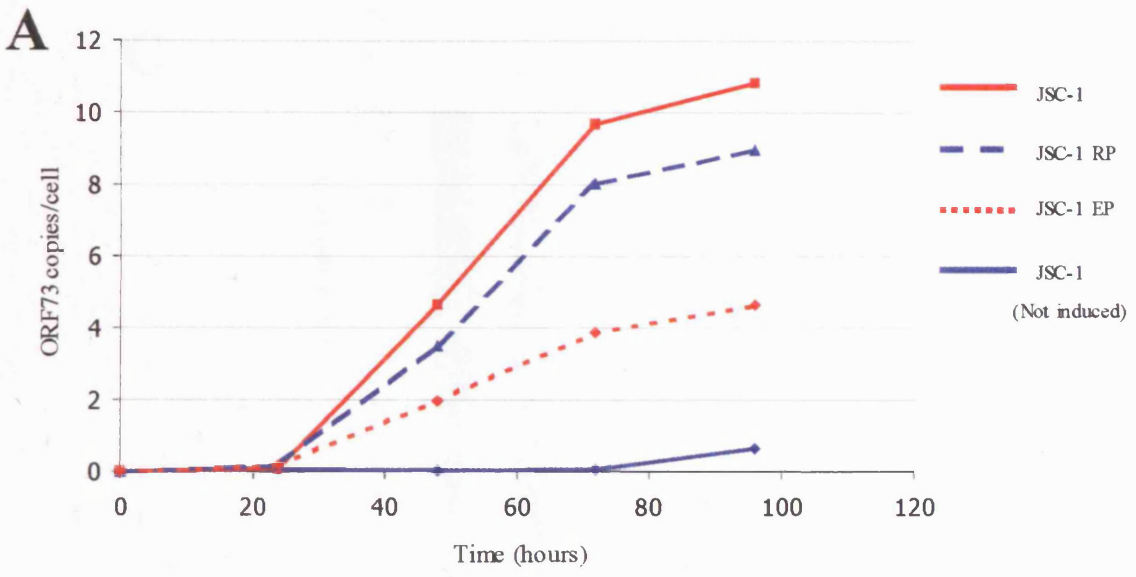


Figure 4.2.3. Expression of lytic proteins downstream of RTA is reduced by shRNA 50E JSC-1 cells transduced or not transduced with shRNA expressing lentiviral vectors were treated with TPA to induce lytic replication. 48-hours post induction cells were fixed and stained for K-bZIP expression. A. Histograms of K-bZIP expression. B. Graphical representation of K-bZIP expression normalised using not transduced TPA induced JSC-1 cells (20.6%). JSC-1 cells transduced or not transduced with shRNA expressing lentiviral vectors were treated with TPA to induce lytic replication. 43-hours post induction cells were fixed and stained for PF-8 expression. Error bars represent the standard error between triplicate experiments. C. Histograms of PF-8 expression. D. Graphical representation of PF-8 expression normalised using not transduced TPA induced JSC-1 cells (20.3%). Error bars represent the standard error between triplicate experiments

JSC-1 cells were transduced with an input equivalent to a MOI 10 on HEK 293-T cells of SJP and EP. Transduced cells were puromycin selected and 8 days postinfection the lytic cycle was induced by TPA treatment for 1-hour, after which the cells were washed 3 times in culture medium, and incubated for either 48 or 42 hours. The time of maximal expression of K-bZIP and PF-8 expression has not been reported and these time points were chosen based on KSHV transcript abundance in JSC-1 cells (Jenner *et al.*, 2001). Following incubation, cells were fixed, permeabilised and stained for K-bZIP or PF-8 and analysed by flow-cytometry. In accordance with figures 4.1.2 and 4.2.2 the number of TPA-induced cells expressing KSHV early gene products was reduced by approximately 75% in the presence of shRNA-50E. This reduction is similar to the reduction in cells expressing RTA in the presence of shRNA-50E and suggests that cells that are able to overcome the RNAi with ORF50 expression faithfully progress through the lytic cycle, whereas cells where RTA expression is blocked fail to enter the lytic cycle and do not express early lytic antigens. It is interesting to note that whilst the number of cells expressing PF-8 is reduced by shRNA-50E expression, the quantity of PF-8 per cell is increased by shRNA-50E expression. The average mean fluorescence intensity (MFI) of PF-8 positive shRNA-50E transduced cells is 32.49 units, more than twice the average MFI of control shRNA transduced and non-transduced cells, 15.04 and 15.51 units respectively. The reasons for this dysregulated PF-8 expression are not clear.

4.2.4. Analysis of virus production

To confirm that shRNA-50E blocks the entire KSHV lytic cycle, we investigated the affect of shRNA-50E on KSHV virus production. To achieve this we utilised a KSHV qPCR assay developed by Dimitra Bourboulia (Bourboulia *et al.*, 2004). This assay utilises a FAM-TAMRA Taq Man probe to quantify the copy number of ORF73 DNA. To adapt this protocol to detect virion-associated DNA in tissue culture supernatants, viral DNA released from dead cells had to be removed prior to extraction of virion-associated DNA. This was achieved by DNase treatment of tissue culture supernatants as previously described (McKnight *et al.*, 2001). Following DNase treatment, virion associated, DNase resistant DNA could be quantified by qPCR. To validate the DNase treatment, culture medium spiked with $5x10^6$ copies of ORF73 DNA was DNase treated and extracted in parallel with experimental samples to ensure efficient DNase treatment.



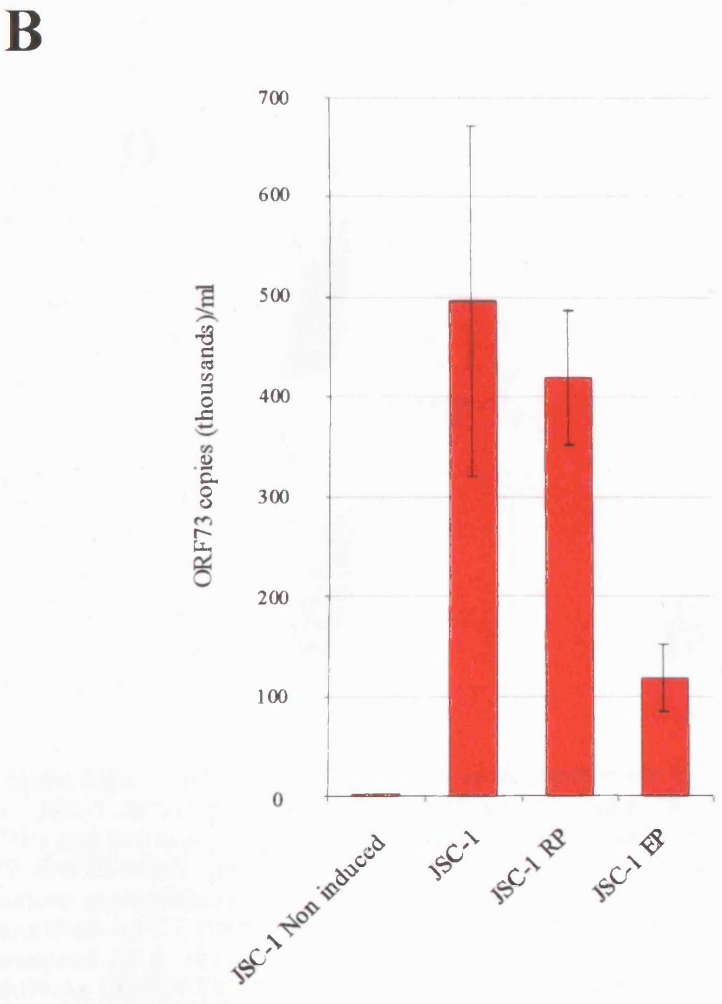


Figure 4.2.4. shRNA ORF50 reduces virus production from TPA induced cells (continued overleaf).

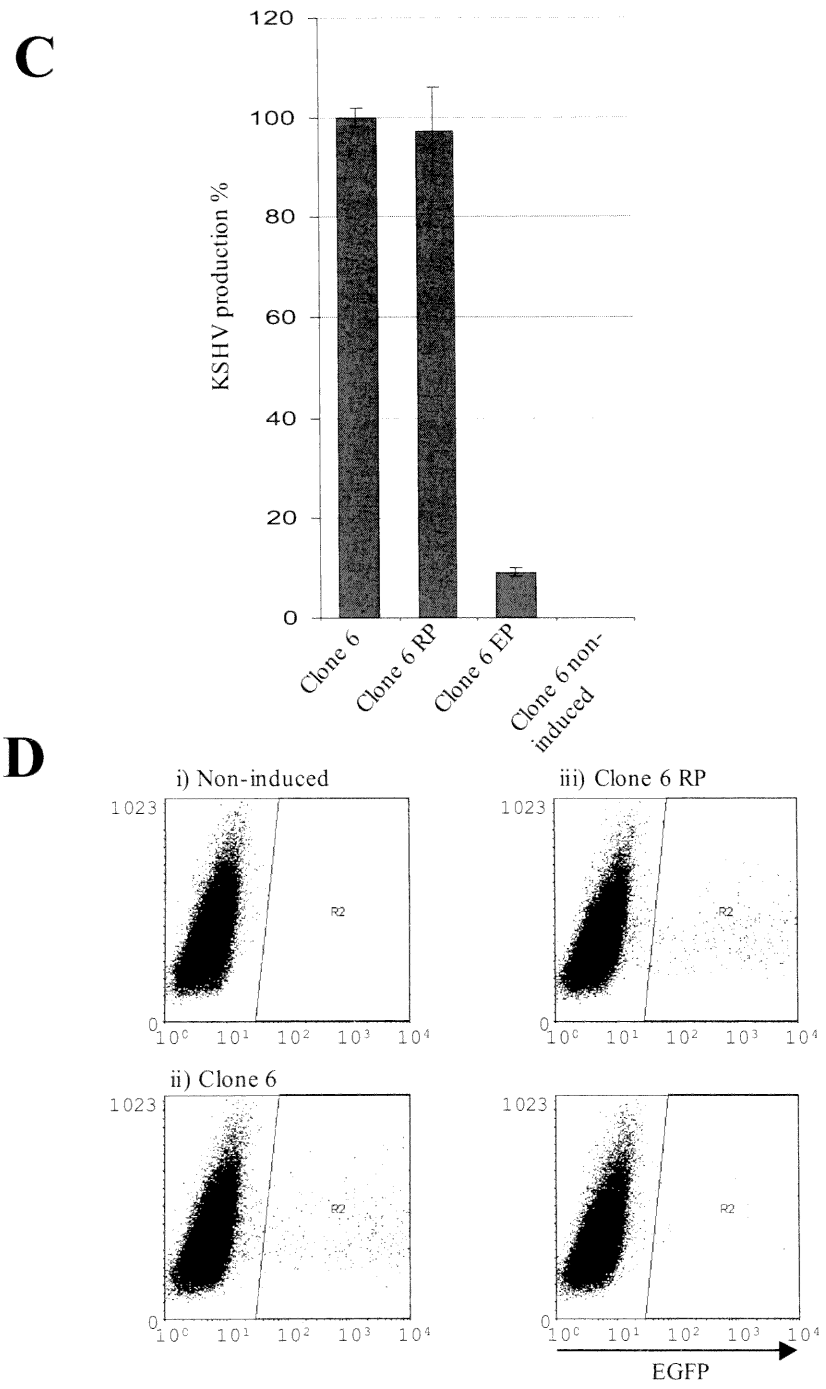


Figure 4.2.4. shRNA ORF50 reduces virus production from TPA induced cells.
A. JSC-1 cells transduced with lentiviral vectors expressing shRNAs ORF50 E (EP) or DsRed (RP) and untreated JSC-1s were seeded at $1x10^5$ cells/ml and treated with TPA. At 0, 24, 48, 72 and 96-hours postinduction DNA was extracted from samples of cell free DNase treated culture supernatants. The mean copy number of ORF73 was determined using real-time quantitative PCR and plotted as the number of copies per cell. Non-induced JSC-1s were also analysed as a control. B. $5x10^4$ JSC-1 cells transduced with lentiviral vectors expressing shRNAs ORF50 E (EP) or DsRed (RP) and untreated JSC-1s were seeded in triplicate. The mean copy number of ORF73 was determined as in A for each replicate and plotted as ORF73 copies/ml. Error bars represent the standard error between triplicate experiments. C. $1x10^7$ HEK 293-T BAC36 clone 6 cells not infected, infected with a MOI 5 of EP or infected with a MOI 5 of RP were treated with TPA to induce lytic replication. Supernatants were harvested, after 72-hours, and used to spinoculate HEK 293-T cells. 48-hours later the number of GFP-positive, BAC36 infected cells was assessed by flow-cytometry. The percentage of virus production is normalised to TPA-induced clone 6 cells (~$1x10^3$ infectious units per ml). Error bars represent the standard error between triplicate experiments. D representative dot plots of the data plotted in C.

JSC-1 cells were transduced with an input equivalent to a MOI 10 on HEK 293-T cells of RP and EP. Transduced cells were puromycin selected and 8 days postinfection the lytic cycle was induced by TPA treatment. Immediately following the addition of TPA, time zero samples were taken. 1-hour following the TPA-induction, cells were washed 3 times in culture medium and incubated for 24, 48, 72 and 96-hours. Following incubation, cell free tissue culture supernatants were stored at -80°C for subsequent analysis. All supernatants were DNase treated and their DNA extracted in parallel. The number of viral DNA copies per ml was determined in triplicate for each time point and an average copy number was determined. Due to the affect of lentiviral vector transduction and TPA treatment on cell doubling times, this figure was adjusted for cell density and plotted as the number of ORF73 copies per cell (figure 4.2.4 A). To examine the reproducibility of this assay, the experiment was repeated in triplicate and ORF73 copy numbers were determined in triplicate for each experimental replicate 72-hours post TPA induction (figure 4.2.4 B).

The results of this investigation suggest that shRNA-50E does block TPA-induced KSHV production from JSC-1 cells. These data suggest that preventing KSHV ORF50 expression with shRNA-50E prevents TPA-induced entry into the lytic cycle, preventing virus production. Those cells which overcome RNAi with ORF50 expression and express RTA seem competent to complete the lytic cycle and produce KSHV virions. This accounts for the reduced levels of virion associated DNA generated by TPA-induced, EP transduced JSC-1 cells.

To confirm the qPCR data we investigated the affect of shRNA-50E on infectious recombinant KSHV production using clone 6 producer cells (described in chapter 5). Clone 6 producer cells are a clonal HEK-293-T derived cell line harbouring infectious recombinant KSHV BAC36 (Zhou *et al.*, 2002). BAC36-infected cells express EGFP driven by the CMV IE promoter. Clone 6 producer cells were transduced with a MOI 5 of RP and EP. Transduced cells were puromycin selected and 14 days postinfection, $1x10^7$ transduced and non-transduced cells were seeded in triplicate. 24-hours later the lytic cycle was induced by TPA treatment and 72-hours postinduction supernatants were harvested and spinoculated onto HEK 293-T cells to quantify BAC36 infection. 48-hours postinfection, HEK 293-T cells were fixed and EGFP-positive cells were quantified by flow cytometry. RNAi with ORF50 expression mediated by shRNA-50E

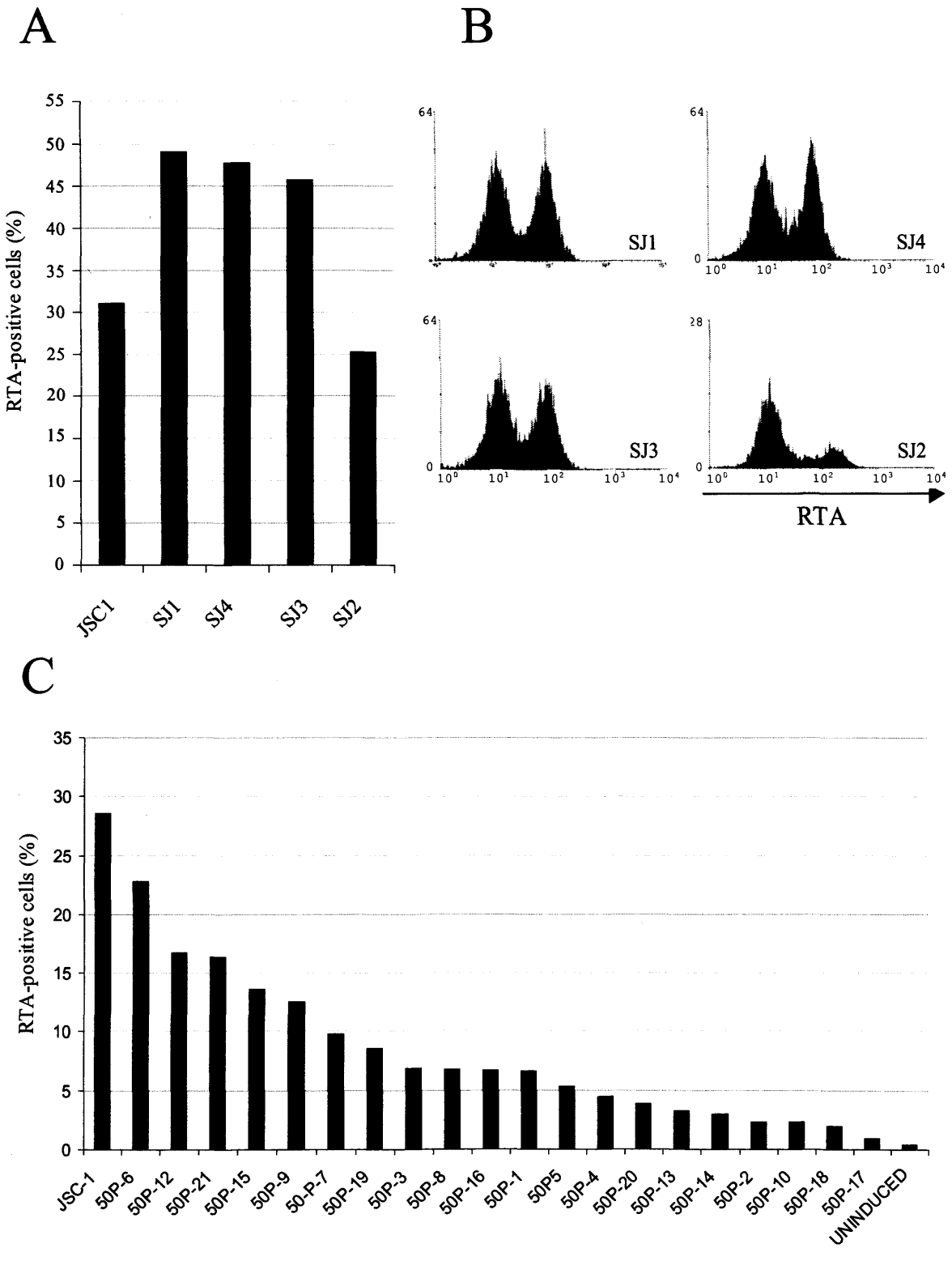


Figure 4.2.5. Clonal analysis of shRNA ORF50.
A. Clonal lines of JSC-1 cells transduced with the lentiviral vector SJP were generated by limiting dilution. 6 weeks later the percentage of RTA-positive cells following treatment with TPA was determined by flow cytometry. B. Histograms of RTA expression depicted in A. C. Clonal lines of JSC-1 cells transduced with the lentiviral vector EP were generated by limiting dilution. 6 weeks later the percentage of RTA-positive cells following treatment with TPA was determined by flow cytometry.

reduced infectious recombinant KSHV production by approximately 90% (figure 4.2.4 C). This effect is specific to shRNA-50E and suggests that shRNA-50E prevents RTA expression, preventing initiation of the lytic cycle resulting in decreased recombinant KSHV production.

4.2.5. Generation of stable knock down cell lines

To investigate the potential of our shRNA expression system to achieve sustained shRNA expression we generated clonal transduced cell lines by limiting dilution. JSC-1 cells were transduced with an input equivalent to a MOI 10 on HEK 293-T cells of SJP and EP. Transduced cells were puromycin selected and 5 days postinfection single cell cultures were seeded. 4 weeks later, 4 SJP and 20 EP clonal populations were expanded and 7 weeks postinfection lytic induction was examined. The KSHV lytic cycle was induced through TPA treatment and 24-hours later cells were fixed, permeabilised and stained for KSHV RTA expression. RTA expression was analysed by flow cytometry and the results are displayed in figure 4.2.5. SJP transduced cell lines were no less responsive to TPA-induction of the KSHV lytic cycle than parental JSC-1 cells. Surprisingly, SJP transduced clonal lines tended to be more responsive to TPA-induction than parental JSC-1 cells, although it is difficult to generalise with so few clonal lines. Interestingly all 20 clonal lines transduced with EP were less responsive to TPA-induction of the lytic cycle than parental JSC-1 cells and some clonal lines such as 50P-17 were barely responsive to TPA-induction. These data suggest that shRNA expression can be maintained for several weeks *in vitro*.

We are unsure whether the smaller number of SJP transduced clonal lines relative to EP transduced clonal lines is a biological phenomenon related to the different shRNAs or is a result of minor differences in the cell densities used to seed the cloning plates.

Discussion II

4.3. shRNA ORF50 prevents entry into the KSHV lytic replication cycle

The aim of this work was to demonstrate the utility of lentiviral vector-mediated RNAi with KSHV gene expression. With this in mind, we targeted the well-characterised ORF50 mRNA and investigated the affect of RTA 'knock down' on KSHV lytic replication.

No rational shRNA design constraints existed when shRNAs 50SJ, 50M and 50E were designed. In the absence of clear constraints, shRNA target sequences, with 5' guanine bases, were selected by eye. The potency of shRNAs targeting these regions was then tested empirically. Both shRNA 50M and shRNA 50 E were able to prevent RTA expression in approximately 80% of cells (figure 4.1.1). Crucially, this reduction was not seen with the control shRNA-DsRed suggesting this is a specific 'knock down' of KSHV RTA. The target sequence of shRNA 50 E was found to be invariant in all cell lines tested and accordingly shRNA 50E was active in both JSC-1 and BC-3 cell lines (figure 4.2.1). Interestingly, despite fewer BC-3 cells supporting KSHV lytic replication following TPA treatment, the proportion of cells prevented from entering the lytic cycle was strikingly similar (approximately 80%).

It is important when considering these data to remember that the reduced size of the population expressing RTA following TPA-treatment is not directly proportional to the level of 'knock down' mediated by shRNA-50E. To directly measure the 'knock down' in ORF50 expression, ORF50 transcript abundance would have to be analysed in the absence of RTA autoactivation. For example, quantitative RT-PCR of KSHV ORF50, following TPA treatment, in the presence of cycloheximide would indicate the level of 'knock down' achieved by shRNA-50E. We have not determined the potency of shRNA-50E and have instead concentrated on the phenotypic effects of shRNA-50E treatment.

The possibility that shRNA-50E merely delayed rather than prevented the majority of cells entering the lytic cycle led us to examine RTA expression over time. RTA expression in JSC-1 cells expressing shRNA-50E has a similar expression profile to normal JSC-1 cells following TPA treatment (figure 4.2.2). These data indicate that shRNA-50E expression prevents the majority of cells from expressing RTA and

entering the lytic cycle. It is likely that the 'knock down' in ORF50 expression prevents the expression of RTA and subsequent autoactivation of the ORF50 promoter. It is tempting to speculate that those cells in which RTA is expressed, at sufficient levels, following TPA treatment are able to overcome the reduction in expression mediated by shRNA-50E and increase ORF50 expression. This in turn could explain the slightly earlier peak in ORF50 expression (figure 4.2.2) in the minority of cells expressing RTA in the presence of shRNA-50E.

To confirm that prevention of ORF50 expression prevents entry into the lytic replication cycle, we analysed KSHV early protein expression (figure 4.2.3). The population of cells expressing K-bZIP and PF-8 was reduced by approximately 75%, very similar to the reduction observed in RTA expression in shRNA-50E expressing cells. Consistent with the accepted role of RTA, this suggests that gene expression downstream of RTA expression is blocked when RTA expression is prevented by shRNA-50E. Interestingly, the expression level of PF-8 seems to be increased in shRNA-50E expressing PF-8-positive cells (figure 4.2.3 panel C). This could represent a dysregulated KSHV gene expression programme in the presence of shRNA-50E, where RTA has escaped RNAi mediated by shRNA50E.

As expected, shRNA-50E reduces the number of DNase resistant genome copies of KSHV Produced by JSC-1 cells and the number of infectious units of BAC36 (described in chapter 5) following TPA treatment (figure 4.2.4).

The generation of multiple clonal lines expressing shRNA-50E that exhibit a silenced phenotype (figure 4.2.5) indicates that shRNA expression can be sustained at effective concentrations for several weeks. Some latently infected JSC-1 cells expressing shRNA 50E express RTA following TPA treatment. Similarly, a varying degree of cells respond to TPA induction in each clonal line.

The increased population of cells expressing RTA in SJP transduced clonal lines was unexpected. It would be interesting to investigate if this phenotype is common in clonal lines generated from non-transduced JSC-1 cells and is stable over multiple passages. Our lentiviral vector-mediated shRNA expression system has subsequently been used to generate stable clonal cell lines with reduced tripartite motif containing protein 5α (TRIM5α) (Ylinen *et al.*, 2005).

The hairpins shRNA-50SJ and shRNA-DsRed have been used interchangeably in this chapter. However, in light of experiments defining the asymmetric loading of the RISC (Schwarz *et al.*, 2003; Khvorova *et al.*, 2003), shRNA DsRed probably represents a more appropriate control, because it is appropriately incorporated into the RISC.

In summary, lentiviral vector-mediated RNAi with ORF50 expression can prevent the majority of cells in a population from expressing RTA. Consistent with the accepted role of KSHV RTA, the block is complete, preventing subsequent gene expression and virion production. Those cells able to overcome the shRNA-50E mediated silencing express RTA, initiate the lytic gene expression cascade and produce new virions. Prevention of lytic replication is the expected phenotype from shRNA-ORF50 indicating our lentiviral vector-mediated shRNA expression system can be effective at interfering with viral gene expression.

Chapter 5

Results: KSHV BAC36 permissivity *in vitro*

5.1. Generating BAC36 producer cells

5.1.1. BAC36

KSHV is a difficult virus to propagate *in vitro* and it was not until 1996 that the first cell culture system for KSHV propagation was reported (Renne *et al.*, 1996b). These systems, involving KSHV from chemically induced PEL cell line culture supernatants, often require greater than 100-fold concentration to achieve substantial infection. More recently recombinant KSHV systems have been developed which can be produced at much higher titres (Zhou *et al.*, 2002) and contain EGFP expression cassettes providing a surrogate marker of infection (Vieira *et al.*, 2001; 2004; Zhou *et al.*, 2002). KSHV BAC36, a generous gift from S. J. Gao, can be used to generate sufficient infectious virions that non-concentrated tissue culture supernatants can be used for infection assays (Zhou *et al.*, 2002). This chapter describes the generation of our BAC36 'producer' cell lines and an investigation into B-cell line permissivity *in vitro*. Although the term permissivity can be used to describe cells supporting lytic viral replication, because KSHV preferentially establishes a latent infection, this chapter uses the term permissivity solely to describe initial cellular infection.

5.1.2. Generating and screening BAC36 'producer' cell lines

The generation of producer cell lines is described in chapter 2. The method used is briefly summarised below and in figure 5.1.2.1. BAC36 DNA was used to transfect HEK 293-T cells. Transfected cells were selected using Hygromycin B. Four weeks posttransfection the cells were treated with TPA to induce lytic replication. Six days later the tissue culture supernatant was harvested, supplemented with polybrene and used to spinoculate $1x10^5$ HEK-293-T cells. 48-hours later the infected cells were selected using Hygromycin B and two weeks postinfection 11 hygromycin resistant colonies were isolated and cultured independently.

To assess KSHV production, $5x10^6$ cells from each clonal line was seeded in a 10cm plate and treated with 20 ng/ml TPA for 1 hour. Six days later the medium was supplemented with 15 μg/ml polybrene and 1 ml was used to spinoculate $1x10^5$ HEK-

293-T cells. Spinoculation involves an inoculation of test supernatants under centrifugation (Forestell *et al.*, 1996). The percentage of EGFP expressing cells was determined by flow cytometry and a graphical representation of the results is show in figure 5.1.2.2 A. Of the 11 clonal lines generated 4 produced substantial amounts of recombinant KSHV (clones 2,5,6 and 9). Clone 6 produced the most infectious virus and was selected as our recombinant KSHV producer cell line.

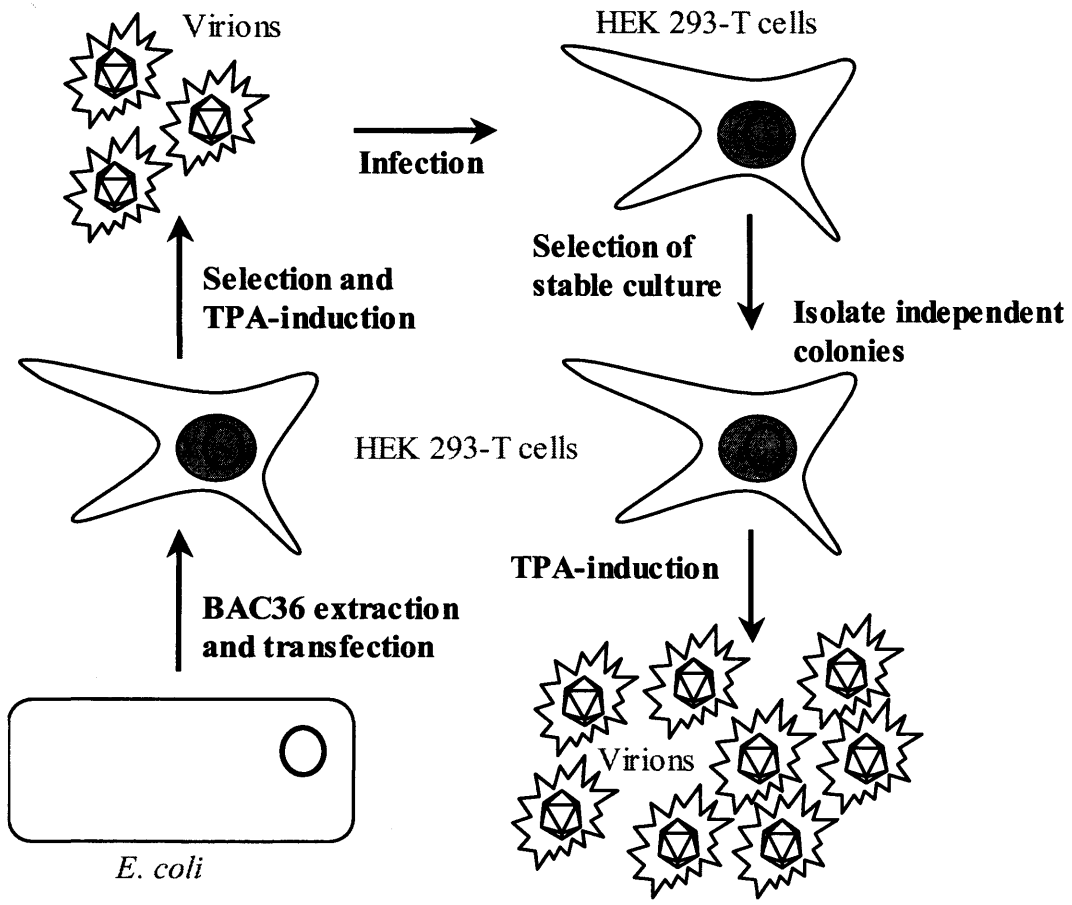


Figure 5.1.2.1. Summary of the method used to generate BAC36 'producer' cell lines
Adapted from Zhou *et al.*, 2002.

To optimise recombinant virus production the amount of infectious virus produced from clone 6 cells, plated at various densities, was assessed over time (figure 5.1.2.2 B). The greatest virus production occurred 72-hours postinduction with TPA at the highest tissue culture density tested ($1x10^7$ cells per 10cm plate). When spinoculated onto $2x10^5$ HEK 293-T cells, supernatant from these conditions resulted in approximately 4.5% infection. This is equivalent to approximately $1x10^4$ infectious units per ml.

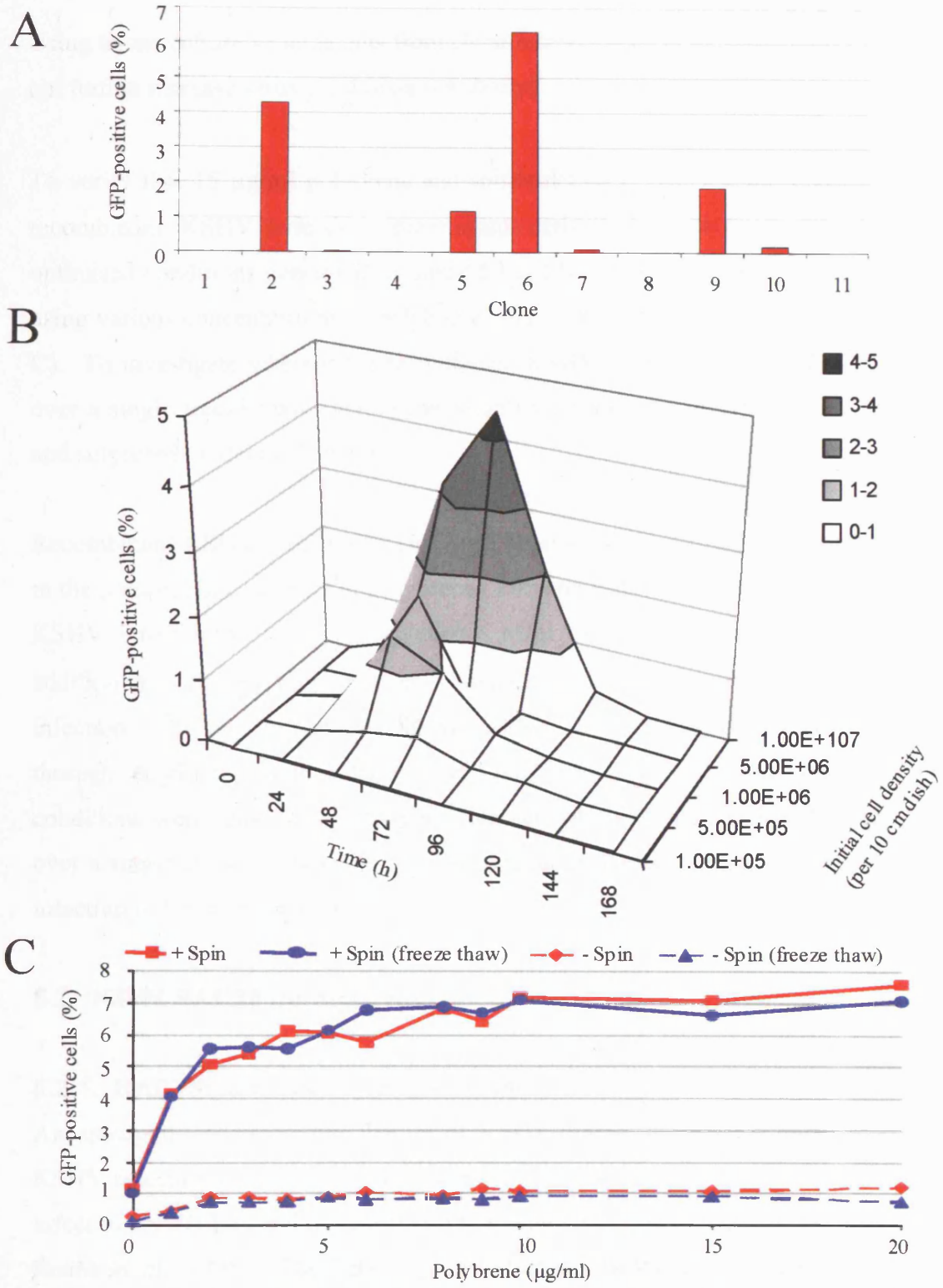


Figure 5.1.2.2. Screening BAC36 'producer' cell lines and optimising virus production
A. $5x10^6$ cells from each clonal line were seeded and lytic replication was induced through treatment with 20ng/ml TPA for 1 hour. 6-days postinduction supernatants were harvested, filtered, supplemented with 15 µg/ml polybrene and used to spinoculate $1x10^5$ HEK 293-T cells. 48-hours postinfection the HEK 293-T cells were fixed and the percentage of EGFP-positive cells was quantified by flow cytometry and is plotted in A. B. Clone 6 cells were seeded at various cell densities, induced as in A and the quantity of infectious recombinant KSHV assessed as in A for various time points postinduction. Clone 6 supernatant was used to infect $1x10^5$ HEK 293-T cells using various concentrations of polybrene in the presence or absence of spinoculation. 48-hours postinfection the HEK 293-T cells were fixed and the percentage of EGFP-positive cells was quantified by flow cytometry and is plotted in C.

Using tissue culture supernatants from clone 6 cells plated at densities above $1x10^7$ did not further increase virus yield (data not shown).

To verify that 15 μg/ml polybrene and spinoculation at 500 x g for 1 hour increased recombinant KSHV infection, supernatant derived from clone 6 cells using the optimised conditions generated in figure 5.1.2.2 B was used to infect HEK 293-T cells using various concentrations of polybrene, with or without spinoculation (figure 5.1.2.2 C). To investigate whether the recombinant KSHV-containing supernatant was stable over a single freeze-thaw cycle, supernatants were frozen and kept at -80°C overnight and subjected to identical analysis.

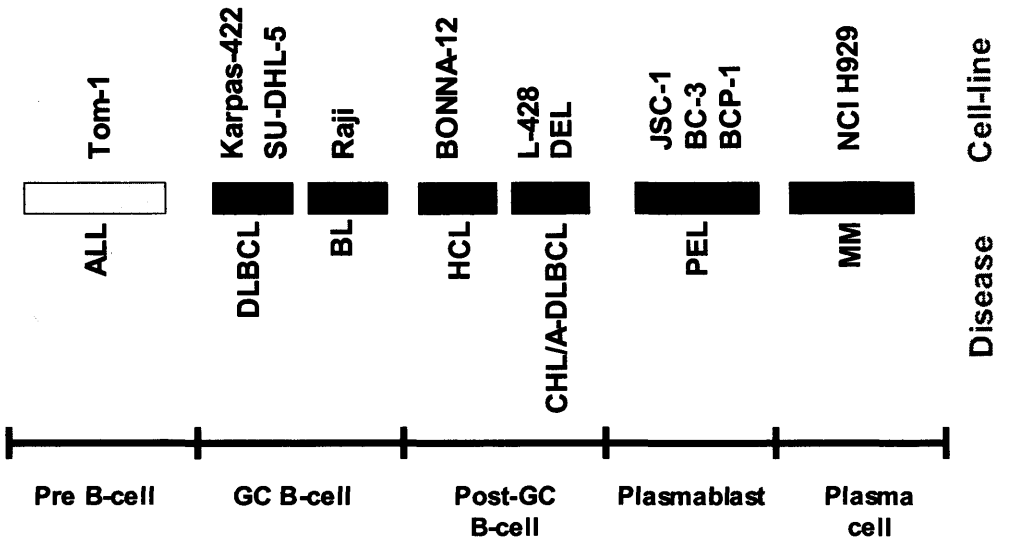
Recombinant KSHV infection is enhanced approximately 7-fold by spinoculation both in the presence and absence of polybrene. Polybrene also clearly enhances recombinant KSHV infection without spinoculation. Most of this enhancement occurs through addition of 2 μg/ml polybrene and higher concentrations only marginally increase infection. The most infection (approximately 7% EGFP-positive cells) was achieved through combining spinoculation with polybrene suggesting our initial infection conditions were reasonably efficient. Reassuringly, recombinant KSHV seems stable over a single freeze thaw cycle, with frozen supernatants resulting in similar levels of infection to fresh supernatants.

5.2. KSHV BAC36 permissivity

5.2.1. BAC36 permissivity in B-cell lines

An apparently contradictory feature of KSHV-biology is that the latent reservoir of KSHV infection is most likely to be B-cells but B-cell lines are notoriously difficult to infect *in vitro* (Chen and Lagunoff, 2005; Bechtel *et al.*, 2003; Blackbourn *et al.*, 2000b; Renne *et al.*, 1998). The EBV negative Burkitt's lymphoma derived cell line BJAB (Menezes *et al.*, 1975) is the only B-cell line in which KSHV infection has been reported *in vitro* (Naranatt *et al.*, 2004; Gasperini *et al.*, 2005). Since the only B-cell lines reported to be recalcitrant to KSHV infection were Burkitt's lymphoma cell lines (Chen and Lagunoff, 2005; Bechtel *et al.*, 2003; Blackbourn *et al.*, 2000b; Renne *et al.*, 1998), we reasoned that B-cell lines derived from malignancies corresponding to different stages of B-cell development might resemble the *in vivo* target of KSHV infection more closely and may therefore be more efficiently infected *in vitro*.

A



B

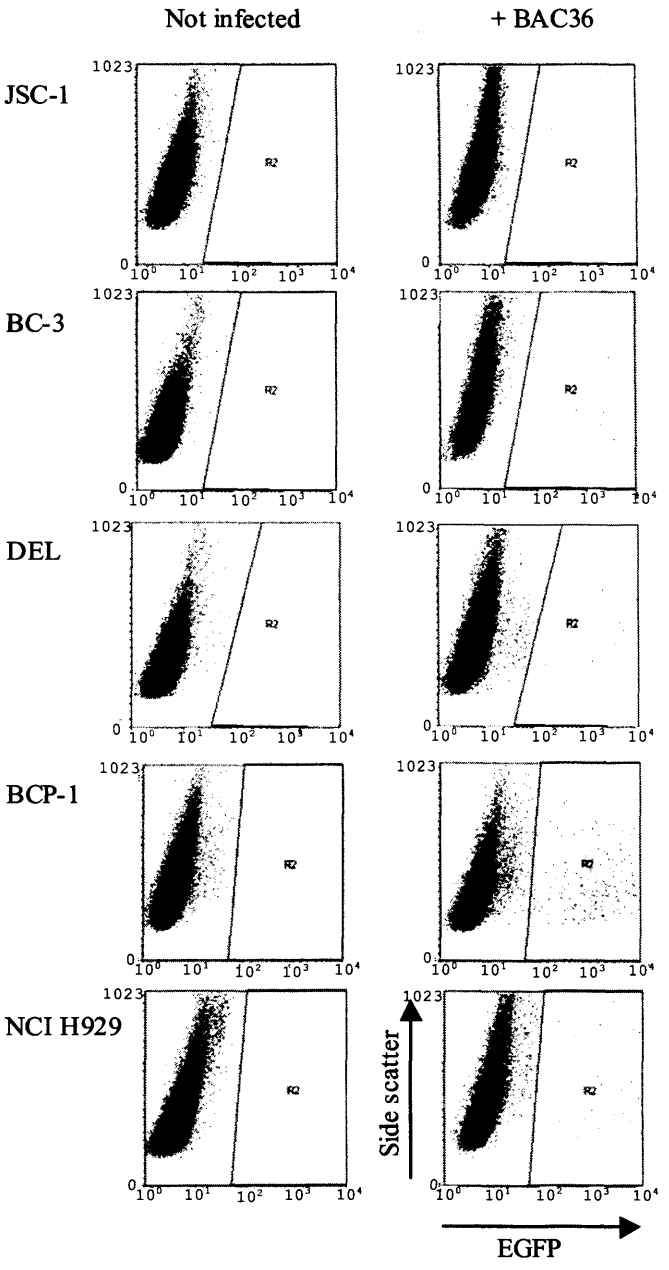
Cell Line	GFP expression (+/-)
TOM1	ND
Karpas-422	-
SU-DHL-5	-
RAJI	-
BONNA12	-
L428	-
DEL	+
BC3	+
JSC1	+
NCI H929	+

C

Cell Line	GFP expression (+/-)
HeLa	++
SLK	++
MUTZ-3	+
HEK 293-T	++
TE 671	++
CRFK	++
FRHK	++
CV-1	++

Figure 5.2.1.1. Recombinant KSHV permissivity *in vitro* (continued overleaf)

D



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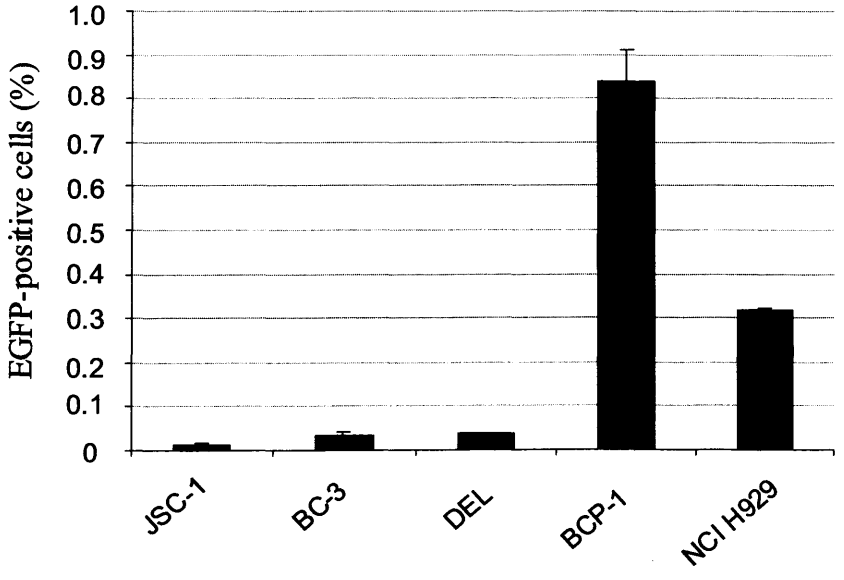
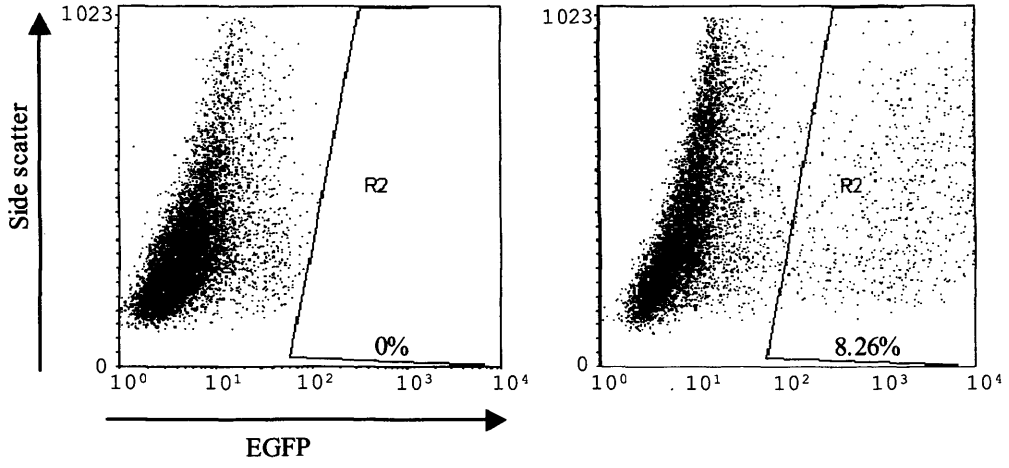


Figure 5.2.1.1. Recombinant KSHV permissivity *in vitro* (continued overleaf)

F



G

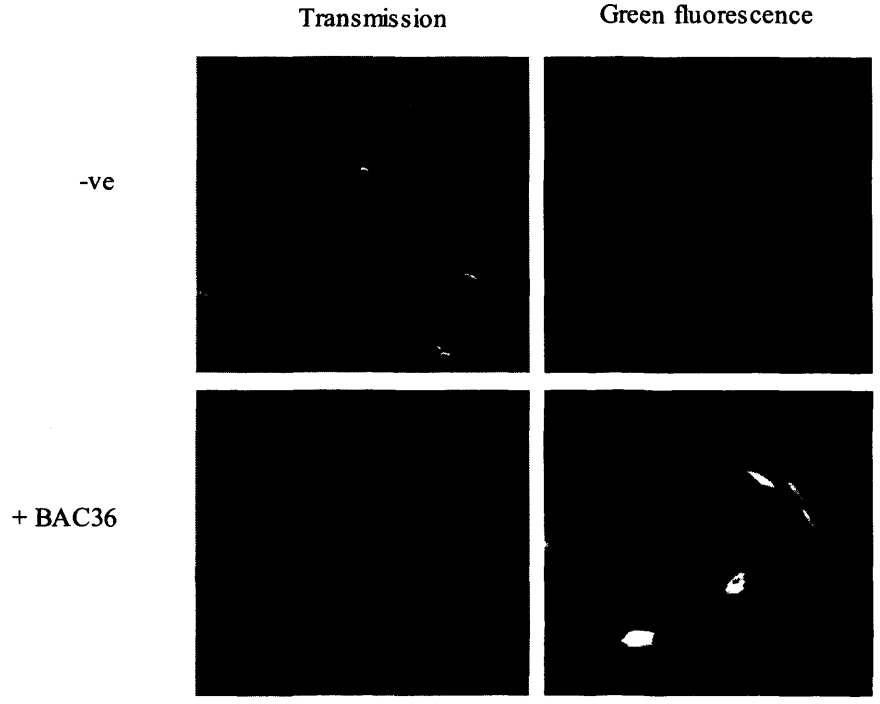


Figure 5.2.1.1. Recombinant KSHV permissivity *in vitro*.
A. A diagrammatic representation of the B-cell lines used in B indicating the disease the cell line was derived from and the developmental stage the cell line represents (Jenner *et al.*, 2003). B the permissivity of B-cell lines to recombinant KSHV infection. $1x10^5$ cells of each cell line were spinoculated with an input equivalent to a MOI 0.1 on HEK 293-T cells. The susceptibility to infection is indicated (- no infection; + <1% EGFP positive cells; ++ >1% EGFP-positive cells ND not determined due to supernatant toxicity). C As in B only a panel of cell lines of non B-cell origin was used. D representative flow cytometry dot plots of the positive cell lines from A. E. Graphical representation of triplicate infections of the cell lines susceptible to infection in A. Error bars represent the standard error between triplicate infections. F. Flow cytometry dot plots indicating efficient infection of primary human nasal chondrocytes spinoculated with an input equivalent to a MOI 0.1 on HEK 293-T cells 48-hours postinfection. G. Representative confocal microscopy fields of chondrocyte infection. B-ALL - precursor B (acute) lymphoblastic leukaemia; DLBCL – diffuse large B-cell lymphoma (GC-like); BL – Burkitt's lymphoma; HCL – hairy cell leukaemia; HL – classical Hodgkin's lymphoma (L-428); A-DLBCL-anaplastic diffuse large B-cell lymphoma; PEL - primary effusion lymphoma; MM – multiple myeloma

To investigate this possibility we assessed the permissivity of a panel of B-cell lines with gene expression profiles similar to different stages of B-cell development (Jenner *et al.*, 2003), which are listed in figure 5.1.1.1 A. $1x10^5$ cells of each cell line were spinoculated with an input of KSHV equivalent to a MOI 0.1 on HEK 293-T cells. The inoculum was removed 6-hours postinfection and the cells were cultured normally. The cell lines were monitored daily for EGFP expression by UV-microscopy. Cell lines we considered to be infected, through UV-microscopic analysis, were analysed by flow-cytometry. Ten days postinfection all cell lines were analysed by flow-cytometry. The results of this analysis are summarised in figure 5.1.1.1 B.

Consistent with previous reports we found B-cell lines largely resistant to KSHV infection. However we did identify two cell lines that could be infected by recombinant KSHV. Both NCI H929 and DEL cell lines were infected at low levels with only 0.3% and 0.03% of cells respectively becoming EGFP-positive 48-hours postinfection. This makes NCI H929 and DEL cell lines approximately 15-fold and 150-fold less susceptible to recombinant KSHV infection than HEK 293-T cells respectively. We were unable to generate latently infected cultures using hygromycin selection (data not shown).

Interestingly, all PEL cell lines tested could be superinfected with recombinant KSHV. The BCP-1 cell line was more than 20-fold more susceptible to recombinant KSHV infection than JSC-1 and BC-3 cell lines.

5.2.2. BAC36 permissivity in a panel of cell lines

To further characterise recombinant KSHV infection *in vitro*, we assessed the ability of KSHV to infect a panel of cell lines commonly used in our laboratory. The results of this analysis are summarised in figure 5.1.1.1 C. Interestingly all adherent cell lines tested were susceptible to recombinant KSHV at levels similar to HEK 293-Ts. The only cell line less susceptible to recombinant KSHV infection was the non-adherent myeloid leukaemia cell line MUTZ-3 (Hu *et al.*, 1996). Consistent with these data primary human nasal chondrocytes, a cell type commonly used in our laboratory (Hidvegi *et al.*, 2005), were also susceptible to recombinant KSHV infection (figure 5.1.1.1 F and G).

Discussion III

5.3. Recombinant KSHV permissivity *in vitro*

The aim of this work was to generate recombinant KSHV 'producer' cell lines for use in assays involving infectious virus. We successfully generated clone 6 'producer' cells and these cells can produce enough infectious recombinant KSHV that unconcentrated supernatants can be used in infection assays.

The magnitude of infection with recombinant KSHV was greatly enhanced through spinoculation and the addition of polybrene. Spinoculation (Forestell *et al.*, 1996) probably increases the rate of virion adsorption without removing the requirement of essential glycoproteins for fusion (Scanlan *et al.*, 2005). This is probably achieved by depositing virions on the surface of target cells thus removing diffusion as a rate-limiting step in virus infection (O'Doherty *et al.*, 2000). Polybrene also increases the rate of virion adsorption (Davis *et al.*, 2002). Polybrene is a polycation and probably increases the rate of adsorption through 'charge shielding' virions from the polyanionic glycosaminoglycans at the cell surface (Davis *et al.*, 2004).

Our investigation of permissivity identified two B-cell lines (NCI-H929 and DEL) which became EGFP-positive following infection with recombinant KSHV. It is important to consider that EGFP expression only indicates that the EGFP expression cassette has reached the nucleus of EGFP expressing cells. In this investigation no analysis of viral gene expression was undertaken and we cannot exclude the possibility that EGFP expression is not a faithful surrogate marker of infection. Indeed, even if the EGFP-positive cells represent genuine infectious events, the level of infection is low enough to suggest that these cells might be no more representative of the favoured B-cell target of KSHV infection than other B-cell lines. However, the level of B-cell line infection does seem to be biased towards later stages of B-cell development. It would be interesting to expand the panel of cells used to see if this relationship holds true.

Every adherent cell line tested was susceptible to recombinant KSHV infection. This was not an unexpected result in light of the ubiquitous expression of heparin sulphate and $\alpha 3\beta 1$ integrins. However the variation in species and lineage of these cell lines makes the recalcitrant nature of B-cell lines to recombinant KSHV even more

intriguing. It is interesting to note that all the adherent cell lines examined where similarly susceptible to recombinant KSHV infection.

Chapter 6

Results: XBP-1 and KSHV reactivation

6.1. XBP-1 in PEL cell lines

6.1.1. Identifying XBP-1s by RT-PCR

PEL cell lines have a plasmablastic immunophenotype and gene expression profile (Jenner *et al.*, 2003; Klein *et al.*, 2003). Because the transcription factor XBP-1 is required for plasma cell differentiation we wanted to determine the splice status of XBP-1 mRNA. XBP-1u is a ubiquitously expressed mRNA which is spliced under conditions of ER-stress. This splicing reaction, catalysed by IRE-1, results in the excision of a 26-nucleotide intron which generates XBP-1s mRNA (Calfon *et al.*, 2002). We adapted a murine XBP-1 PCR assay developed by Heather Harding (Calfon *et al.*, 2002) and assessed the ability of our PCR assay to distinguish between XBP-1s and XBP-1u cDNA.

Oligonucleotides 13 and 14 were used as primers to amplify a region of cDNA spanning the cryptic XBP-1 intron. Using XBP-1u as a template, this generates a 249 bp product that can be cleaved by *Pst* I digestion. Using XBP-1s as a template, PCR amplification generates a 223 bp product that is resistant to *Pst* I digestion (Figure 6.1.1 A). When both templates are used together, a third, *Pst* I resistant, product is apparent when visualised by agarose gel electrophoresis. This third band is resistant to *Pst* I digestion and migrates just below the 300 bp marker. To confirm that this third band was a hybrid between XBP1u and XBP1s PCR products, the *Pst* I resistant band was excised, gel purified and sequenced. Analysis of sequence chromatograms indicate that the sequence of this band is XBP-1 until the 5' splice site is reached. Five nucleotides into the intron it is clear that multiple templates are present in the sequencing reaction (Figure 6.1.1 C). The divergence is not apparent until 5 nucleotides into the intron because the first 5 nucleotides downstream of the intron are identical to the first 5 nucleotides of the intron. Analysis of the 3' sequence identifies dual Taq-derived A-overhangs, 26 nucleotides apart, the same length as the cryptic XBP-1 intron (Figure 6.1.1 D). These data confirm that the third band is a PCR-hybrid and that both Pst I resistant bands require the presence of XBP-1s for a template.

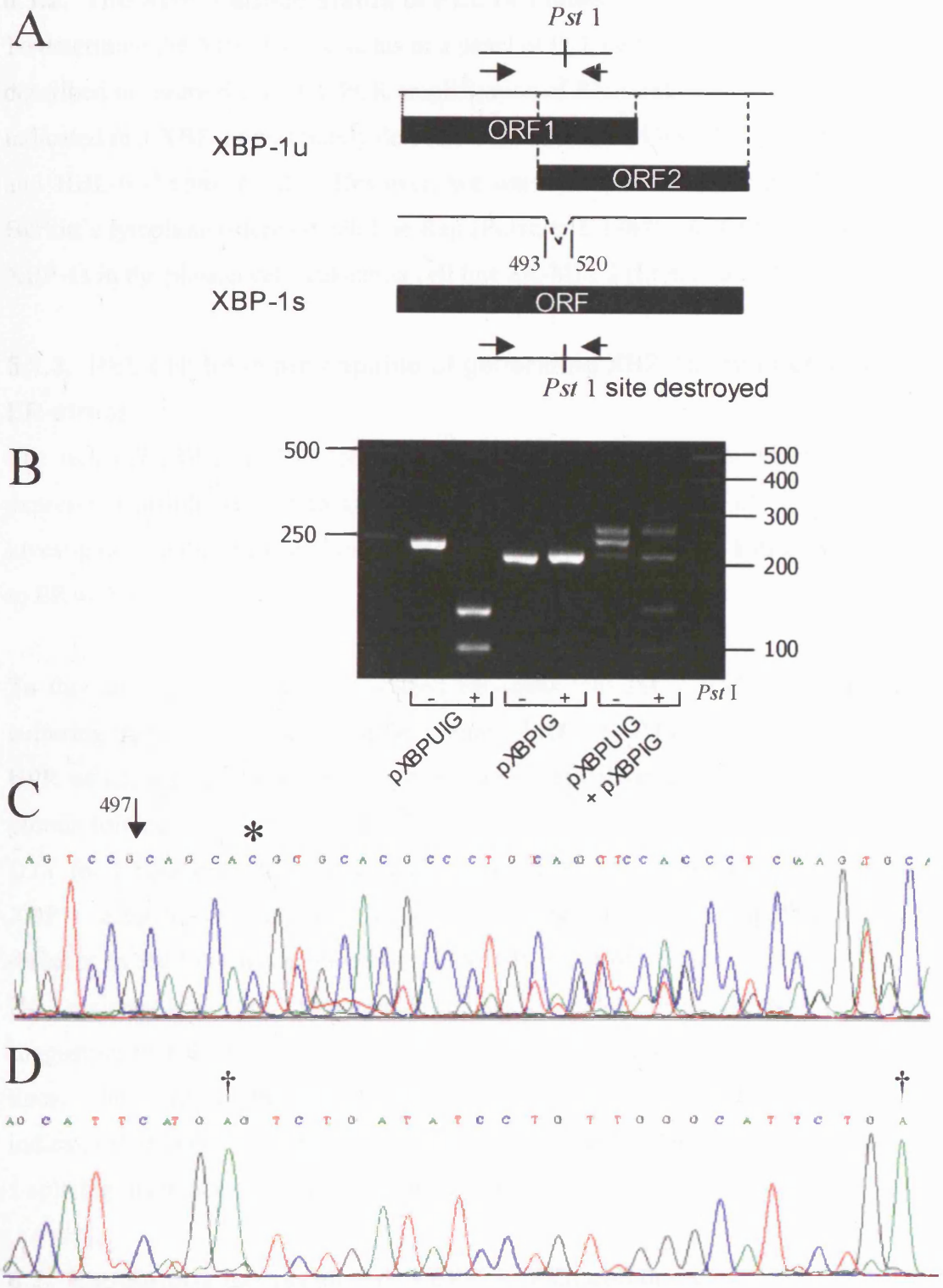


Figure 6.1.1. XBP-1 RT-PCR
A. A diagrammatic representation of human XBP-1 mRNA. Nucleotides are numbered relative to translation initiation. The naturally occurring *Pst* I recognition sequence is indicated. B. PCR using cDNA templates of XBP-1s (pXBPIG) and XBP-1u (pXBPUIG) as templates. PCR products were incubated in the presence or absence of the restriction endonuclease *Pst* I prior to agarose gel-electrophoresis. Numbers indicate the length in base pairs of DNA standards. C Sequencing chromatogram of the gel-purified highest molecular weight band of *Pst*-1 digested PCR product using a mixture of pXBPIG and pXBPUIG as a template. The 5' splice junction (497) and the beginning of divergent sequence is indicated (*). D. As in C with the Taq-derived A-overhang from both PCR products highlighted (†) spaced by exactly 26 nucleotides.

6.1.2. The XBP-1 splice status of PEL cell lines

To determine the XBP-1 spice status of a panel of PEL cell lines we used the PCR assay described in figure 6.1.1. RT-PCR amplification of RNA extracted from PEL cell lines indicated that XBP-1s was barely detectable in the PEL cell lines BC-3, BCBL-1, JSC-1 and HBL-6 (Figure 6.1.2). However, we were easily able to detect XBP-1s in the Burkitt's lymphoma-derived cell line Raji (Pulvertaft, 1964) and substantial amounts of XBP-1s in the plasma cell leukaemia cell line SK-MM-2 (Eton *et al.*, 1989).

6.1.3. PEL cell lines are capable of generating XBP-1s under conditions of ER-stress

The lack of XBP-1s in PEL cell lines, even relative to Raji cells which have a gene expression profile similar to germinal centre B-cells (Jenner *et al.*, 2003), led us to investigate whether PEL cell lines were capable of splicing XBP-1 mRNA in response to ER-stress.

To this end, we artificially introduced ER-stress into JSC-1 and BC-3 cell lines by culturing them in the presence of Dithiothreitol (DTT). DTT is a potent inducer of the UPR which acts as a reducing agent inhibiting disulphide bond formation and correct protein folding. JSC-1 and BC-3 cells were cultured normally or in the presence of 2 M DTT for 1-hour prior to RNA extraction and cDNA synthesis. PCR amplification of XBP-1 using oligonucleotides 13 and 14 as primers led to the amplification of DNA resistant to *Pst* I digestion only from cDNA derived from cells treated with DTT. The *Pst* I resistant bands are more apparent than the XBP-1u, *Pst* I sensitive, PCR product suggesting that the stress-induced splicing of XBP-1u mRNA is efficient in PEL cell lines. The ability of PEL cell lines to splice XBP-1u mRNA in the presence of DTT indicates that the machinery required to sense ER-stress and the, IRE-1-mediated, XBP-1 splicing machinery are intact in these cell lines.

6.2. Exogenous expression of XBP-1 in PEL cell lines

6.2.1. IE, XBPIG and XBPUIG

To investigate the affect of XBP-1s expression on KSHV reactivation, we exogenously expressed XBP-1s in PEL cell lines. Because of the difficulty of transfecting PEL cell lines this was achieved using lentiviral vectors. All the vectors used in this chapter contain an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES)

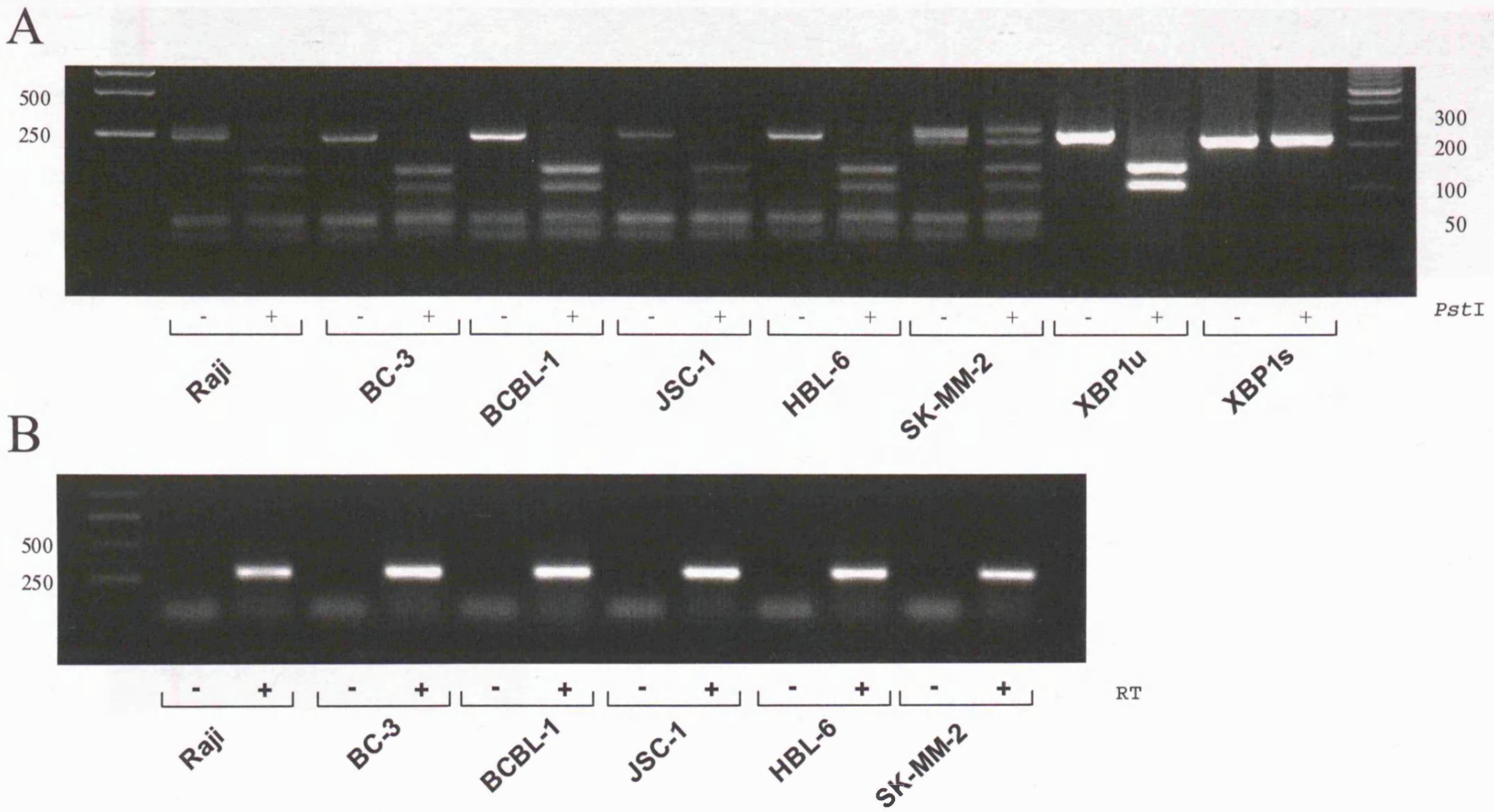


Figure 6.1.2. The XBP-1 splice status of PEL cell lines

A. RNA was extracted from a panel of PEL cell lines, Raji cells and SK-MM-2 cells. The RNA was used to synthesize cDNA using an oligo dT primer and used as a template in the XBP-1 PCR assay used in figure 5.1.2. PCR products were incubated in the presence or absence of the restriction endonuclease *Pst* I prior to agarose gel-electrophoresis. XBP-1u and XBP-1s standards were also amplified. Numbers indicate the length in base pairs of DNA standards. B. XBP-1 PCR amplification using cDNA synthesis reactions with and without the addition of reverse transcriptase as templates.

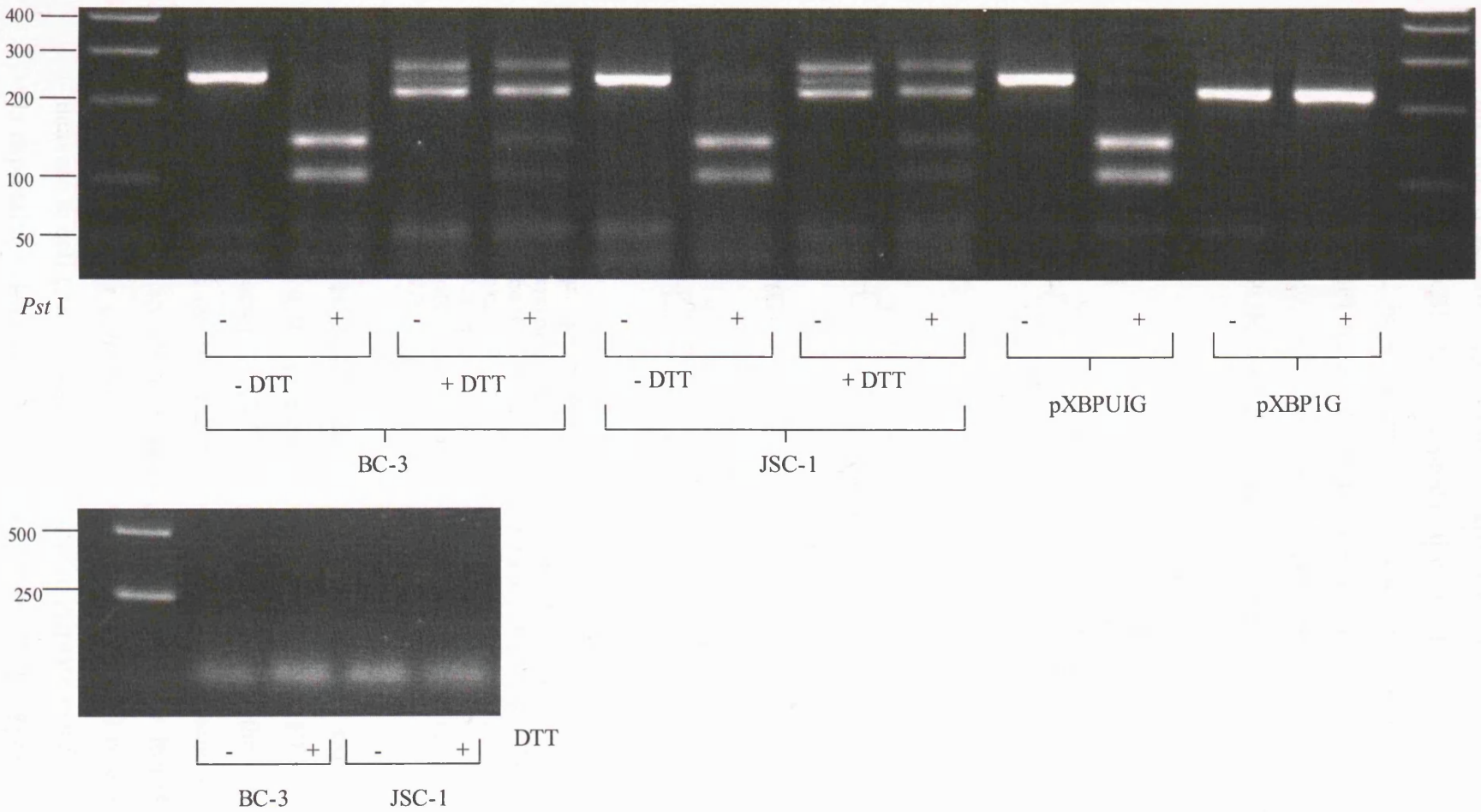


Figure 6.1.3. PEL cell lines are capable of generating XBP-1s in response to ER-stress
A. JSC-1 and BC-3 cells were cultured in the presence or absence of 2 M DTT. RNA was extracted and used to synthesize cDNA using an oligo dT primer. This cDNA was used as a template in the XBP-1 PCR assay used in figure 5.1.2. PCR products were incubated in the presence or absence of the restriction endonuclease *Pst* I prior to agarose gel-electrophoresis. XBP-1u and XBP-1s standards were also amplified. Numbers indicate the length in base pairs of DNA standards. B. XBP-1 PCR amplification using cDNA synthesis reactions without the addition of reverse transcriptase as templates.

which mediates translation of an EmGFP cistron. The lentiviral vector genome plasmid pIE was a generous gift from Y. Ikeda and the lentiviral vector genome plasmids pXBPIG and pXBPUIG were produced by C. Tsantoulas, an MSc student under my supervision. IE is an 'empty vector' encoding only the IRES-EmGFP transcript. XBPIG and XBPUIG encode XBP-1s and XBP-1u cDNA cistrons upstream of the IRES respectively, and express XBP-1 and EmGFP following transduction. The IE, XBPIG and XBPUIG vector-genomes are summarised diagrammatically in figure 6.2.1.

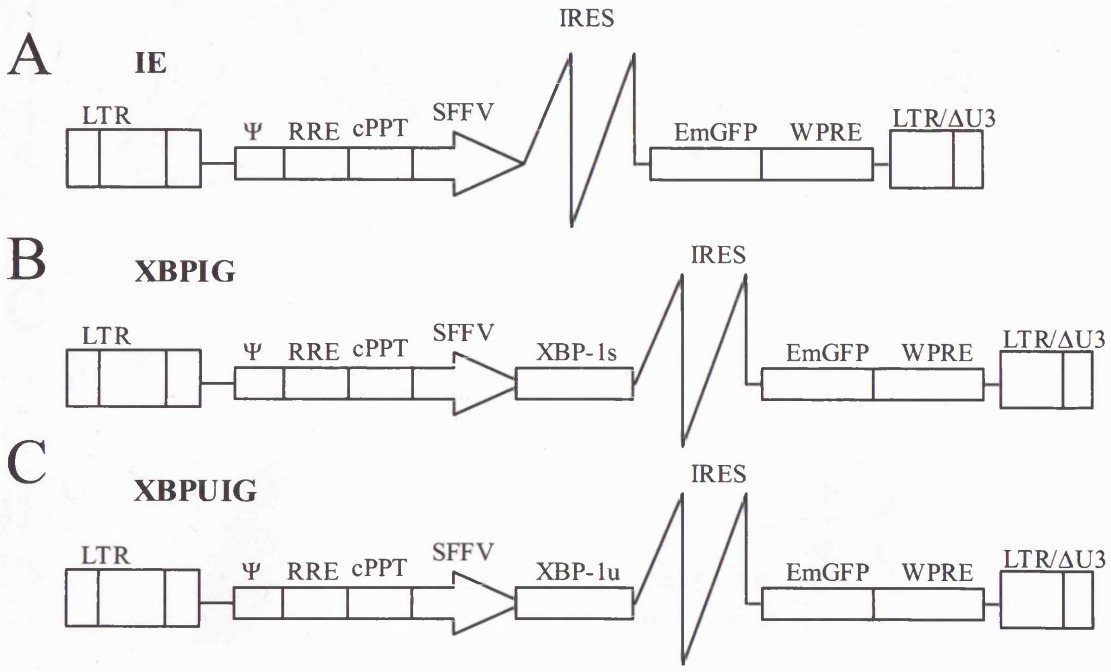


Figure 6.2.1. XBP-1 RT-PCR
A diagrammatic representation of IE (A), XBPIG (B) and XBPUIG (C) lentiviral vector-genomes. Long terminal repeat (LTR), retroviral packaging signal (Ψ), Rev response element (RRE), central poly pyrimidine tract (cPPT), internal spleen focus forming virus LTR (SFFV), coding sequence for XBP-1 (XBP-1u/XBP-1s), internal ribosome entry site (IRES), coding sequence for Emerald GFP (EmGFP) Woodchuck hepatitis virus polypyrimidine rich element (WPRE) and a self inactivating LTR (LTR/ΔU3).

To confirm the expression and activity of exogenously expressed XBP-1, JSC-1 cells were transduced with an input equivalent to a MOI 5 on HEK 293-T cells of IE, XBPIG and XBPUIG vectors. XBP-1s expression results in the expansion of the secretory apparatus, which can be quantified by flow cytometry using side scatter as a measure of cellular granularity (Shaffer *et al.*, 2004). Although lentiviral vector transduction increases cellular granularity, transduction with XBPIG results in a greater than 60% increase in cell granularity. This substantial increase in side scatter suggests that XBP-1s expression is leading to expansion of the secretory apparatus.

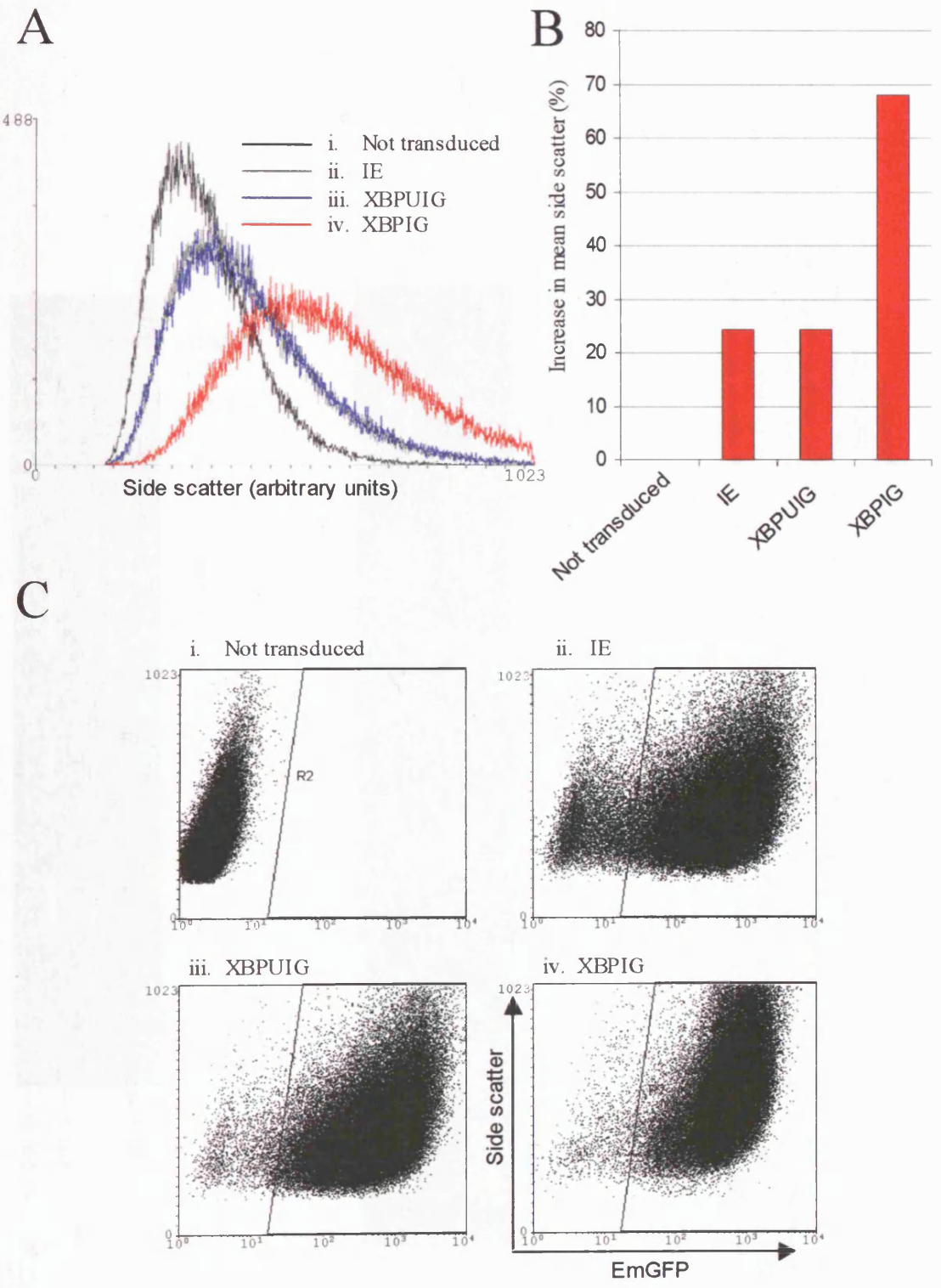


Figure 6.2.2. XBPIG transduction of JSC-1 cells results in expansion of the secretory apparatus and XBP-1s mRNA expression (continued overleaf).

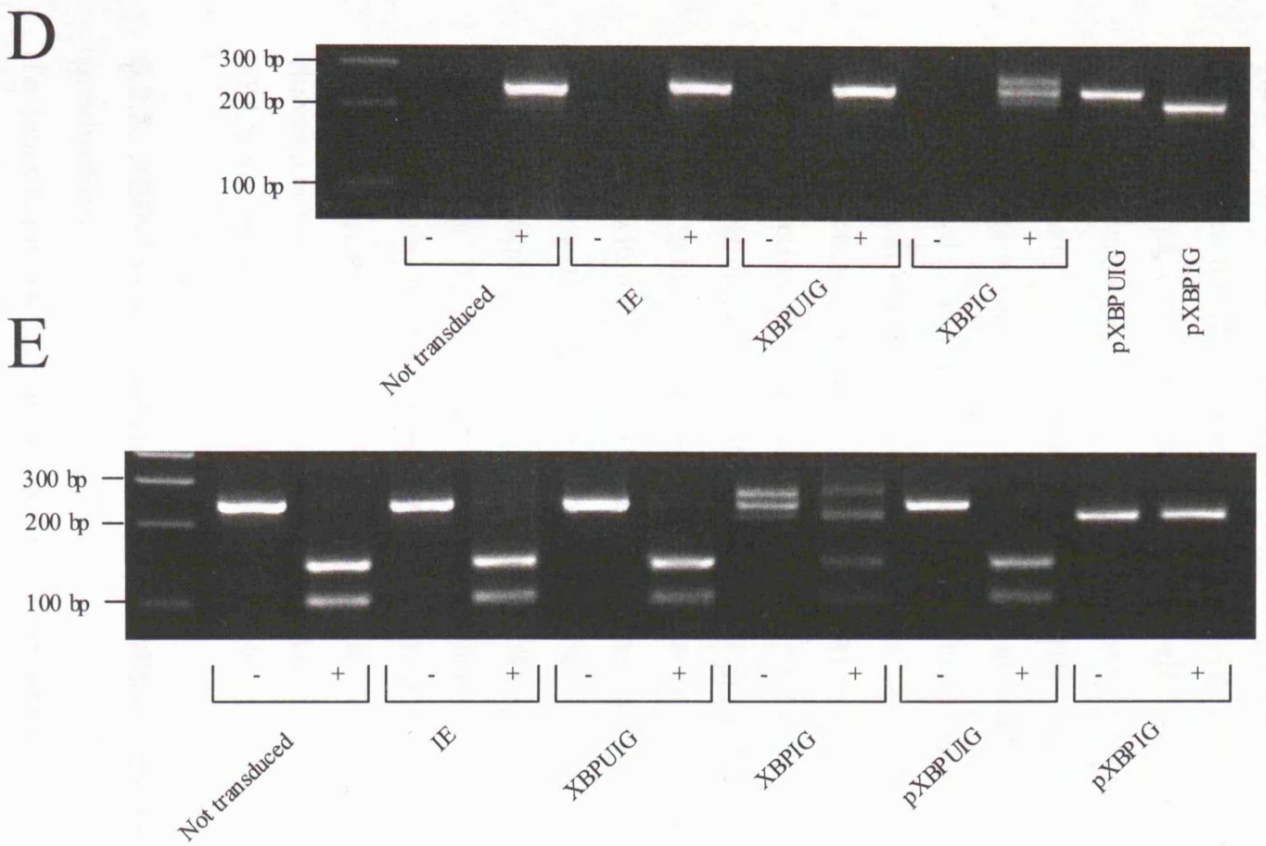


Figure 6.2.2. XBPIG transduction of JSC-1 cells results in expansion of the secretory apparatus and XBP-1s mRNA expression.
A. Side scatter histograms of JSC-1 cells, 48-hours post infection, transduced with an input equivalent to a MOI 5 of IE, XBPIG and XBPUIG lentiviral vectors or not transduced. B A graphical representation of the mean side scatter of the histograms in A. C. Dot-plots from the same samples depicted in A indicating EmGFP fluorescence and side scatter. D. RNA was extracted from the samples in A and used to synthesize cDNA using an oligo dT primer which was used as a template in the XBP-1 PCR assay used in figure 5.1.2. PCR amplification using cDNA synthesis reactions with and without the addition of reverse transcriptase as templates is shown. E. The PCR products shown in D were incubated in the presence or absence of the restriction endonuclease *Pst* I prior to agarose gel-electrophoresis. XBP-1u and XBP-1s standards were also amplified in D and E. Numbers indicate the length in base pairs of DNA standards.

RT-PCR analysis of transduced cells indicates that *Pst*-1 resistant DNA can be amplified from XBPIG transduced cells, confirming XBP-1s mRNA expression (figure 6.2.2.E). No amplification was seen using cDNA synthesis reactions without reverse transcriptase indicating the *Pst* I resistant bands result from cDNA amplification and not amplification from contaminating plasmid or lentiviral vector DNA (figure 6.2.2 E).

6.2.2. XBPIG transduction results in RTA expression

To investigate the affect of exogenous XBP-1s expression on KSHV reactivation, we transduced PEL cell lines with an input equivalent to a MOI 5 on HEK 293-T cells. 48-hours post-transduction cells were fixed, permeabilised and stained for KSHV RTA expression using a monoclonal antibody specific for KSHV RTA (Okuno *et al.*, 2002), a generous gift from Keiji Ueda. TPA treated cells were also stained as a positive control for lytic replication. To allow time for vector penetration, reverse transcription integration and transgene expression, PEL cell lines were treated with TPA 24-hours post-transduction. Because of the wide emission spectrum of EmGFP we used an allophycocyanin (APC) conjugated secondary antibody to detect RTA expression. The excitation and emission spectra of APC and EmGFP are entirely separate (Craig and Carr, 1968) which facilitates the simultaneous measurement of RTA and EmGFP expression without a requirement for fluorescent compensation. Analysis of RTA expression by flow cytometry indicates that XBPIG transduction results in RTA expression (figure 6.2.3). This induction of RTA expression is not observed using the 'empty vector' IE. Crucially, in XBPIG infected cultures only EmGFP expressing cells, transduced with XBPIG, express RTA indicating that induction of RTA expression only occurs in transduced cells. The level of RTA expression, as judged by the mean fluorescence intensity of RTA staining, is reduced in XBPIG transduced cells relative to TPA treated cells. The reasons for and the relevance of this difference are unclear.

6.2.3. XBP-1s expression in clone 6 cells increases recombinant virus production

To investigate whether RTA expression resulting from XBPIG transduction could initiate KSHV lytic replication, we assessed the ability of transfected pXBPIG to increase recombinant KSHV production.

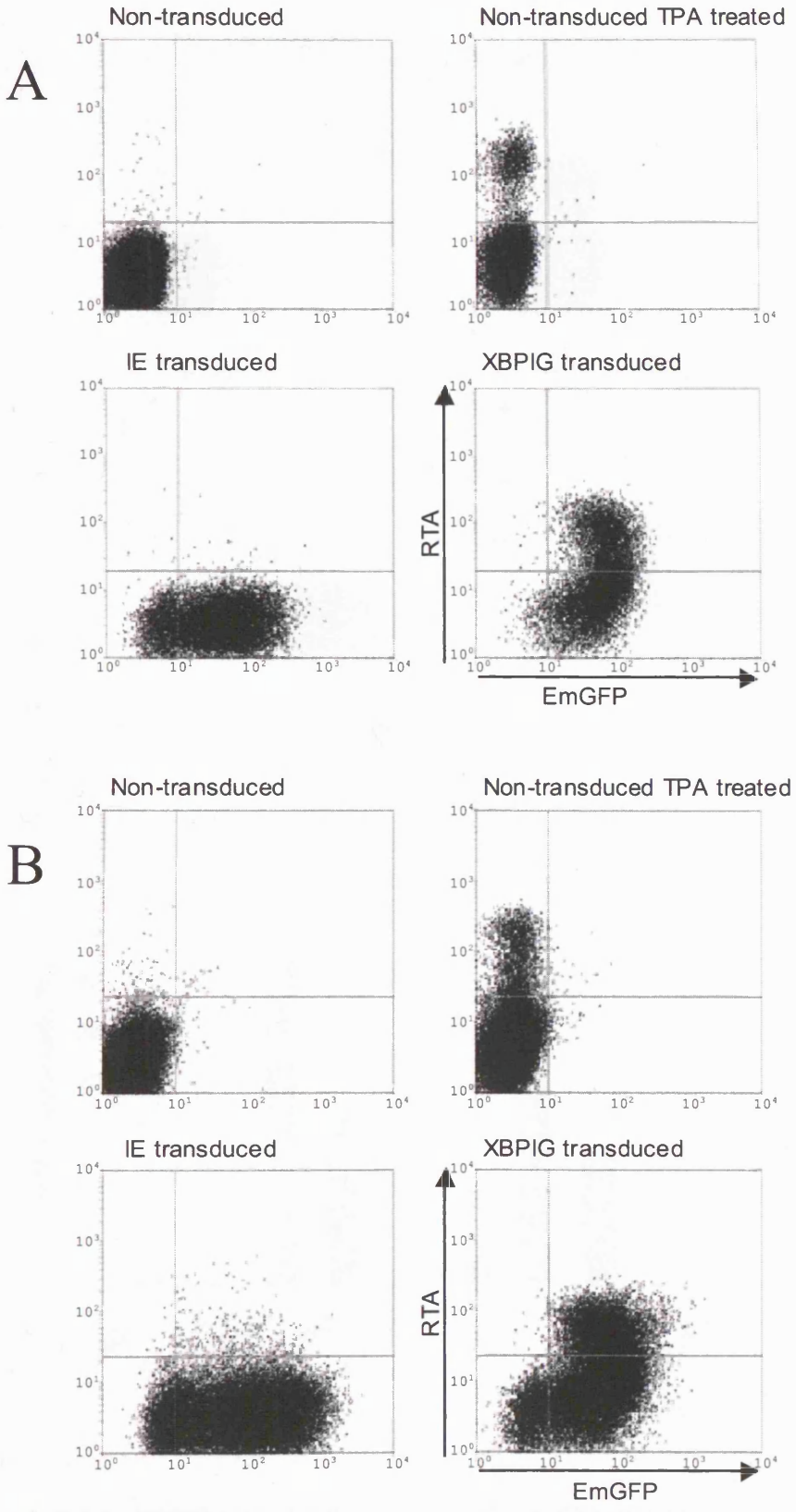


Figure 6.2.3. XBPIG transduction induces KSHV-RTA expression (continued overleaf)

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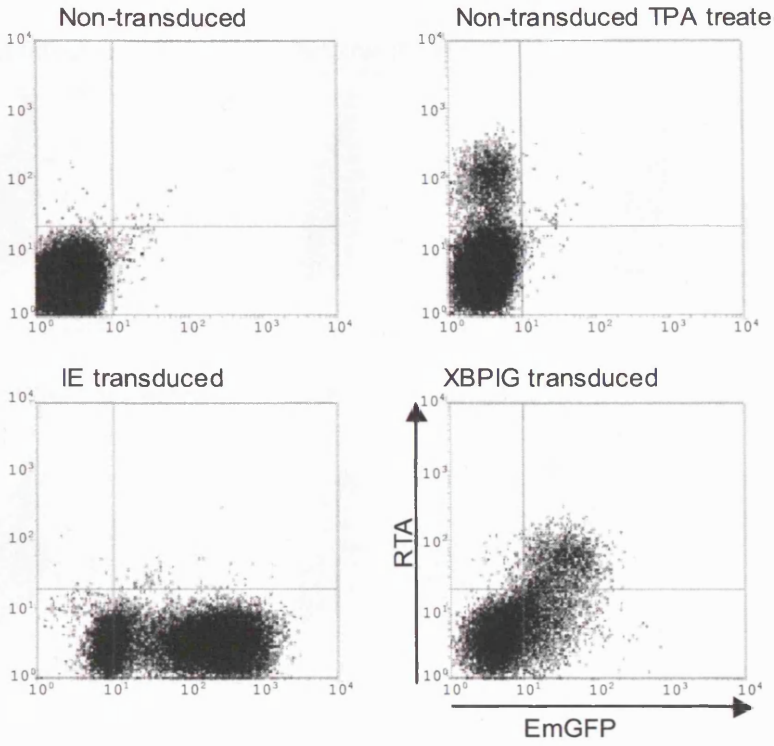

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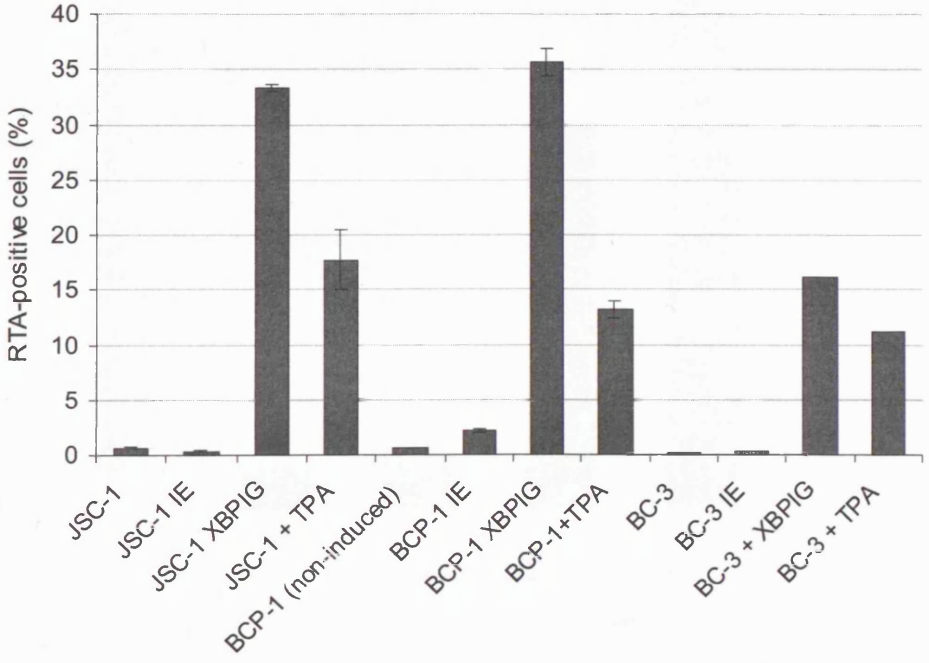
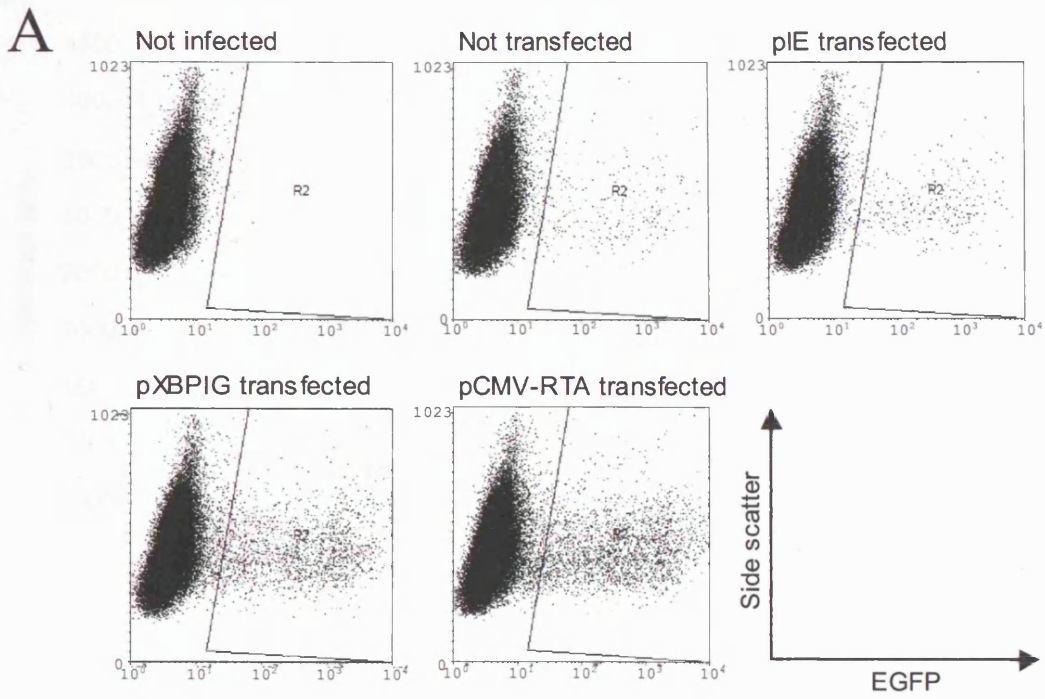


Figure 6.2.3. XBPIG transduction induces KSHV-RTA expression
A. $8x10^5$ JSC-1 cells were transduced with an input equivalent to a MOI 5 of IE and XBPIG lentiviral vectors. 48-hours post-transduction, cells were fixed permeabilised and stained for RTA expression. Non-transduced cells and non-transduced cells treated with TPA for 24-hours were also stained as negative and positive controls respectively. B. As in A using BCP-1 cells. C. As in A using BC-3 cells. D a graphical representation of the dot plots in A, B and C. Error bars represent the standard error between triplicate experiments.



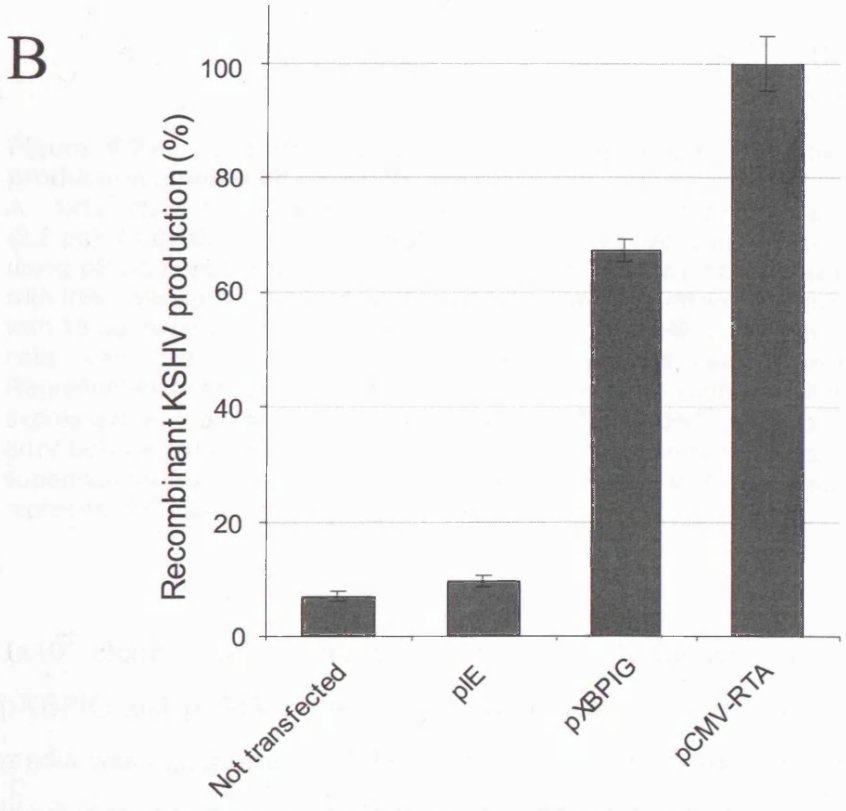


Figure 6.2.4. pXBPIG transfection of clone 6 cells increases recombinant KSHV production (continued overleaf)

C

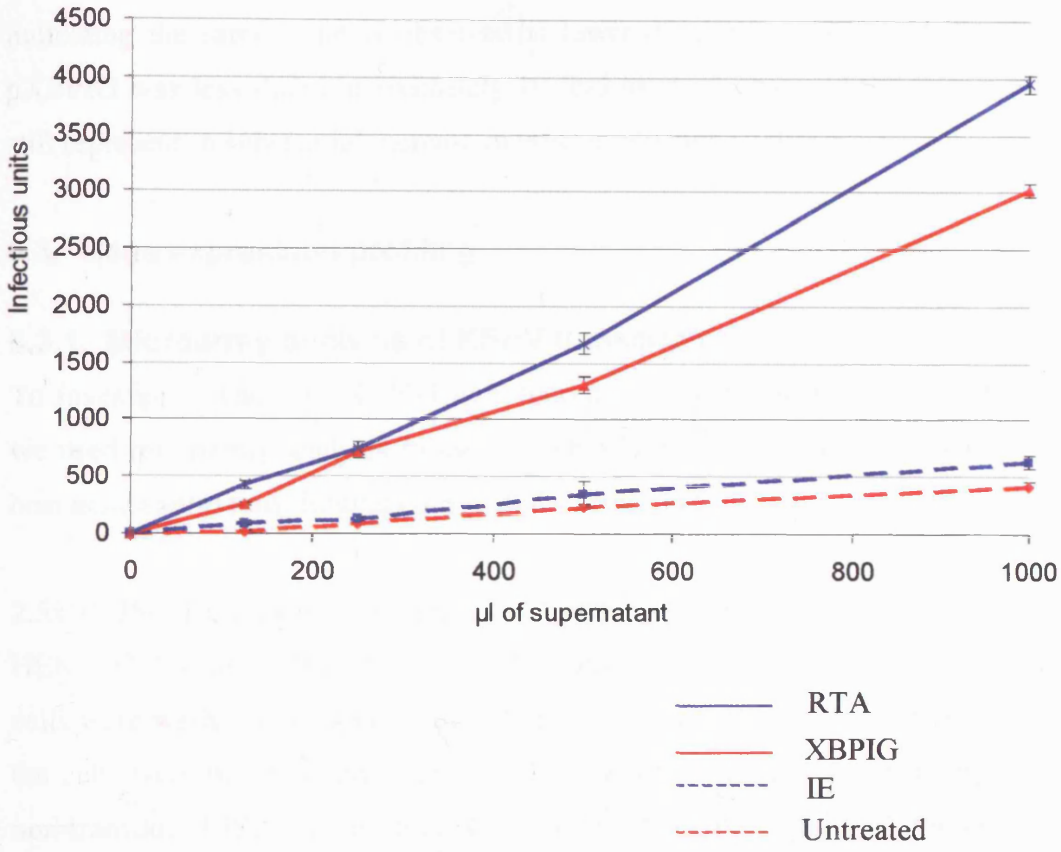


Figure 6.2.4. pXBPIG transfection of clone 6 cells increases recombinant KSHV production (continued overleaf)
A. $1x10^7$ clone 6 cells were seeded and transfected in triplicate with molar equivalents of pIE (2.5 µg), pXBPIG (2.8 µg) and pCMV-RTA (1.7 µg). All transfections were made up to 2.8 µg using pBluescript II KS +. 24-hours posttransfection the medium was removed and replaced with fresh medium. 72-hours posttransfection the medium was removed, filtered, supplemented with 15 µg/ml polybrene and used to spinoculate $2x10^5$ HEK 293-T cells. 48-hours postinfection cells were fixed and the number of EGFP-positive cells quantified by flow cytometry. Representative dot-plots are shown. B. Graphical representation of the dot plots in A expressed as a percentage relative to pCMV-RTA (100%). Error bars represent the standard error between triplicate experiments. C. 2-fold serial dilution of recombinant KSHV containing supernatants from a single sample of the experiment in A repeated in triplicate. Error bars represent the standard error between triplicate repeats.

$1x10^7$ clone 6 cells were transfected in triplicate with molar equivalents of pIE, pXBPIG and pCMV-RTA (a generous gift from A. Whitehouse). 24-hours later the media was replenished and the cells were incubated for a further 48-hours. Following incubation recombinant KSHV containing supernatants were filtered, supplemented with 15 µg/ml polybrene and used to spinoculate $2x10^5$ HEK 293-T cells. 48-hours postinfection the number of EGFP-positive cells was determined by flow cytometry. Transfection of clone 6 cells with pXBPIG resulted in a greater than 6-fold increase in recombinant KSHV production relative to nontransfected and pIE transfected controls

(figure 6.2.4 A and B). This analysis was done using undiluted supernatants and a 2-fold dilution series of recombinant KSHV-containing supernatants is also shown indicating the same trend is observed at lower dilutions. The 6-fold increase using pXBPIG was less than approximately 10-fold increase observed for pCMV-RTA, but still represents a substantial increase in virus production (figure 6.2.4).

6.3. Gene expression profiling

6.3.1. Microarray analysis of KSHV transcripts

To investigate whether pXBPIG transduction causes lytic reactivation in JSC-1 cells, we used microarray analysis to assess global KSHV gene expression and a subset of host gene expression simultaneously on the same chip.

$2.5x10^6$ JSC-1 cells were transduced in triplicate with an input equivalent to a MOI 5 on HEK 293-T cells of IE, XBPUIG and XBPIG vectors. 6-hours post-transduction the cells were washed once and resuspended in fresh medium. 72-hours post-transduction, the cells were pelleted and resuspended in TRIzol reagent. Contemporary samples of non-transduced JSC-1 cells and JSC-1 cells treated with 20 ng TPA for 48-hours were also resuspended in TRIzol reagent. Total RNA was purified from all samples and the quality and quantity assessed using an Agilent bioanalyzer. From this, mRNA was labelled with Cy5 by RT-PCR using a custom primer set containing primers specific to every KSHV ORF (Jenner *et al.*, 2001) in addition to oligo dT primer. Labelled cDNA was mixed with Cy3-labelled reference RNA and hybridised to the KSHV-human microarray. A common reference RNA mixture was used to enable comparison across the whole sample set. The cDNA labelling, array-hybridisation and scanning was performed by Dr Catherine Gale.

The data were examined by cluster analysis to identify patterns in the gene expression data and identify relationships between the samples. Both the genes and samples were ordered using a self-organising map algorithm, which acts to minimise the differences between adjacent nodes. This ordering was then used to control the orientation of the dendrogram nodes subsequently generated by hierarchical clustering (figure 6.3.1). This process distinguishes between the experimental conditions used, separating TPA treatment and XBPIG transduction, which induce KSHV lytic gene expression, from IE transduction and untreated cells which do not express substantial levels of lytic

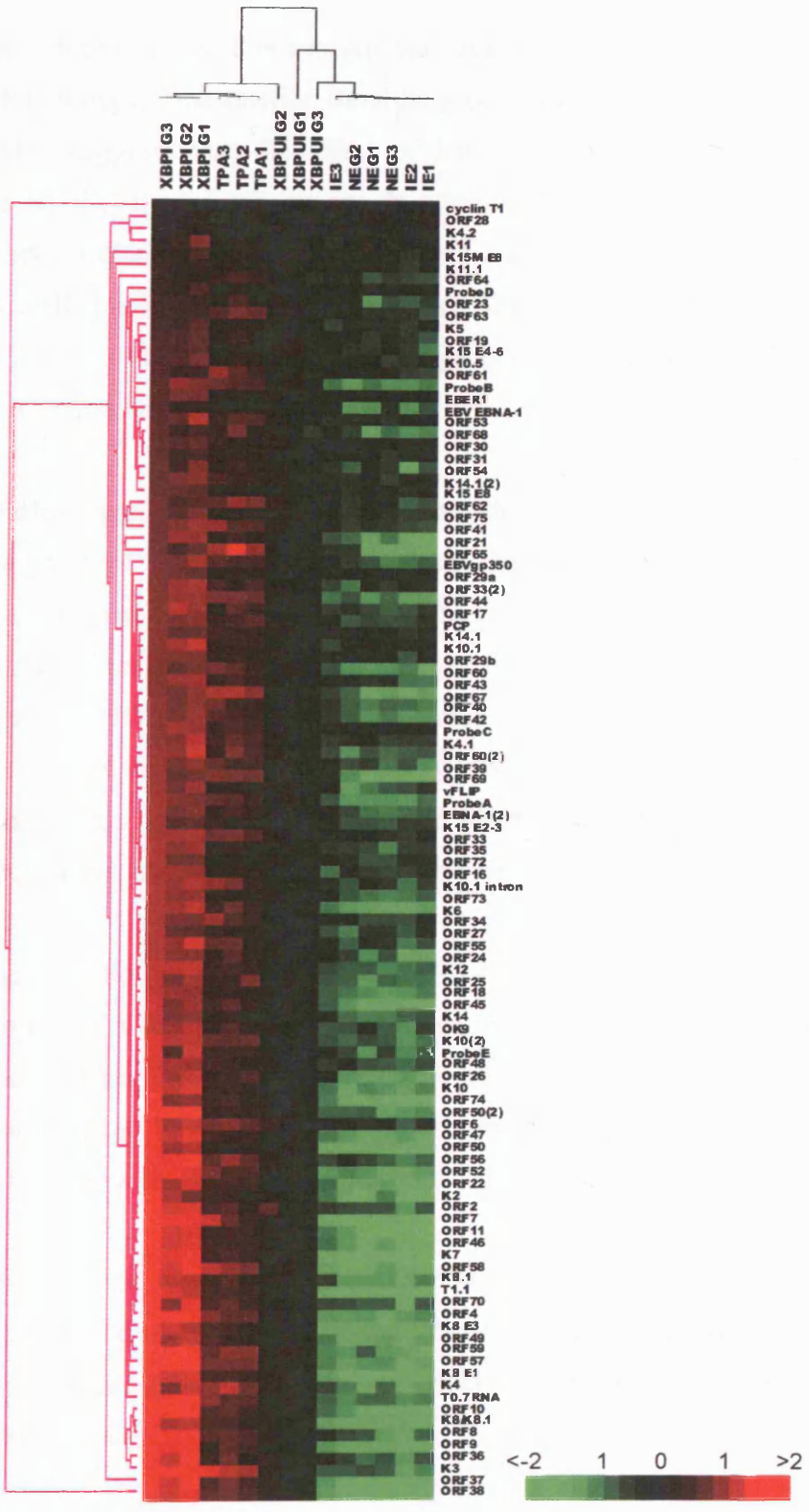


Figure 6.3.1. Hierarchical clustering of 15 samples and a filtered set of 108 genes. Each column represents one sample and each row one gene. The dendrogram on the left represents the relationship between genes in terms of their expression pattern. Gene expression is shown as a pseudo-coloured representation of log(2) expression ratio with red being above and green below the row/column median level of expression (set to 0) as shown by the scale. The cDNA labelling, array hybridisation and scanning was performed by Dr Catherine Gale.

transcripts. Interestingly, this analysis was unable to distinguish the gene expression profiles following IE transduction from the gene expression profiles of untreated JSC-1 cells. This suggests that the affect of lentiviral vector-transduction has minimal influence on JSC-1 gene expression. In addition, XBPUIG transduced cells seem to have an intermediate phenotype with repeats 1 and 3 clustering with IE transduced and untreated cells but repeat 2 clustering with XBPIG-transduced and TPA-treated cells. Figure 6.3.1 shows the cluster of lytic viral genes most strongly upregulated in response to TPA-treatment and XBPIG-transduction.

6.3.2. Fold-regulation of KSHV transcripts

To assess the lytic cycle initiated by XBPIG, XBPUIG and TPA, we determined the fold-change in gene expression for each of the viral transcripts from the cluster in figure 6.3.1 relative to untreated JSC-1 cells and this data is shown in figure 6.3.2. With few exceptions, XBPIG transduced cells expressed the highest level of KSHV transcripts followed by TPA treated cells, followed by XBPUIG transduced cells followed by IE transduced cells. The fold-change trend of XBPUIG and XBPIG transduced cells are extremely similar, and both broadly follow the trend of TPA-induced JSC-1 cells.

A notable exception to this trend are ORFs 61-69, with the exception of ORF66, whose expression in TPA treated cells is more upregulated than in XBPIG transduced cells. It is unclear whether this represents differing gene expression programmes induced by TPA treatment and XBPIG transduction or whether it represents asynchrony between samples induced with TPA and samples induced by XBP-1 expression.

Reassuringly, with the exception of ORF74 (vGPCR), no KSHV transcripts were greater than 1-fold up or downregulated following IE transduction relative to untreated JSC-1 cells. This highlights the minimal affect of lentiviral vector-transduction on JSC-1 gene expression.

6.3.3. Significance analysis of microarray (SAM) analysis

To further quantify the affect of XBP-1 on JSC-1 gene expression, significance analysis of microarray (SAM) analysis was used on this data set. Because the author did not conduct SAM analysis, this data is not considered here. However, for

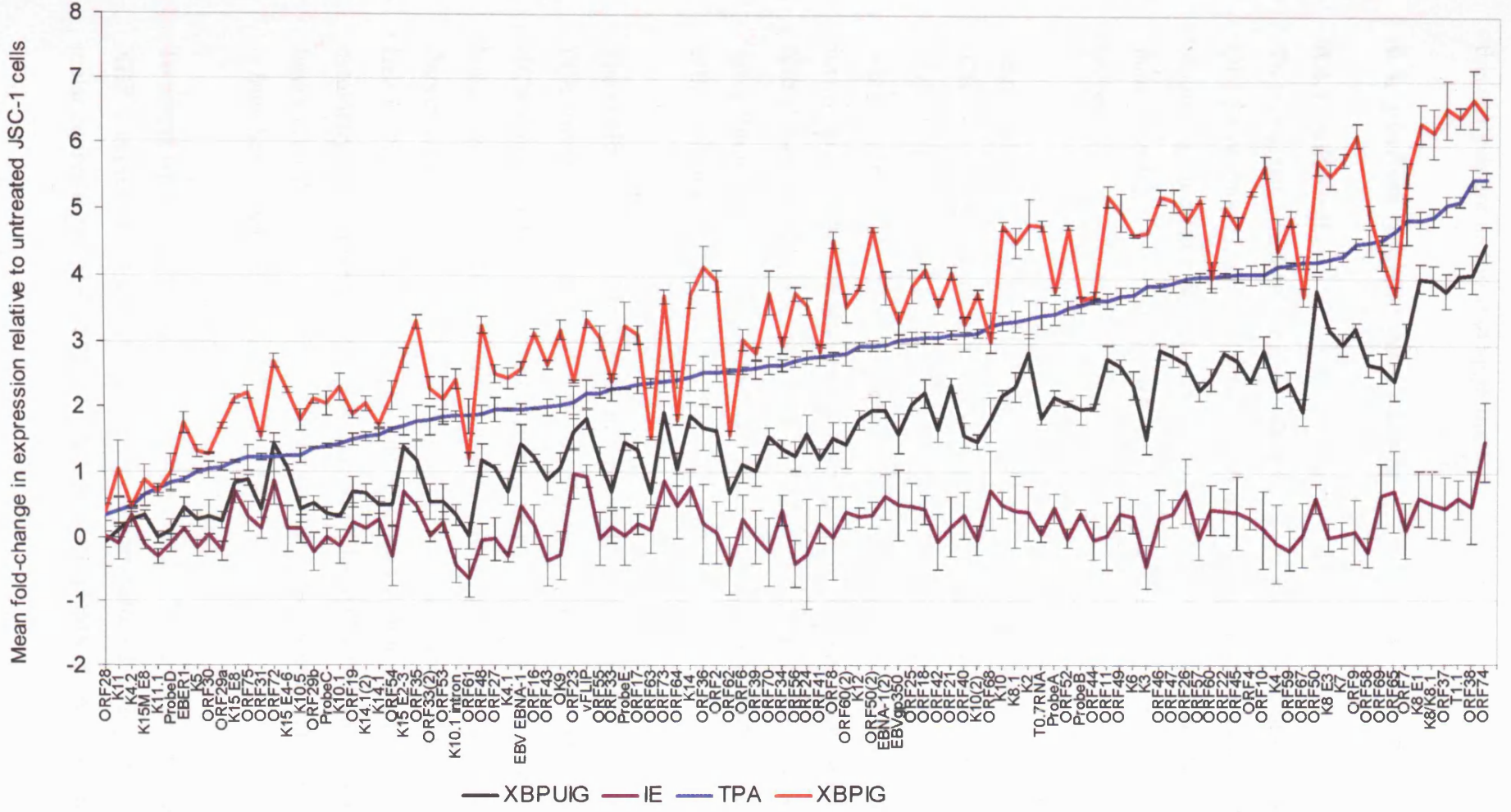


Figure 6.3.2. Fold-change in KSHV transcript abundance
The fold change in KSHV gene expression from the data displayed in figure 6.3.1. Log(2) expression ratios for each gene are plotted relative to untreated JSC-1 cells as a fold-change in transcript abundance. The genes are ordered with respect to increasing fold-change of TPA-treated JSC-1 cells. Error bars represent the standard error between triplicate repeats.

completeness, the method of SAM analysis is described in chapter 2 and the results of this analysis are included in appendix 1.

6.4. p50Redi reporter gene assays

6.4.1. p50Redi

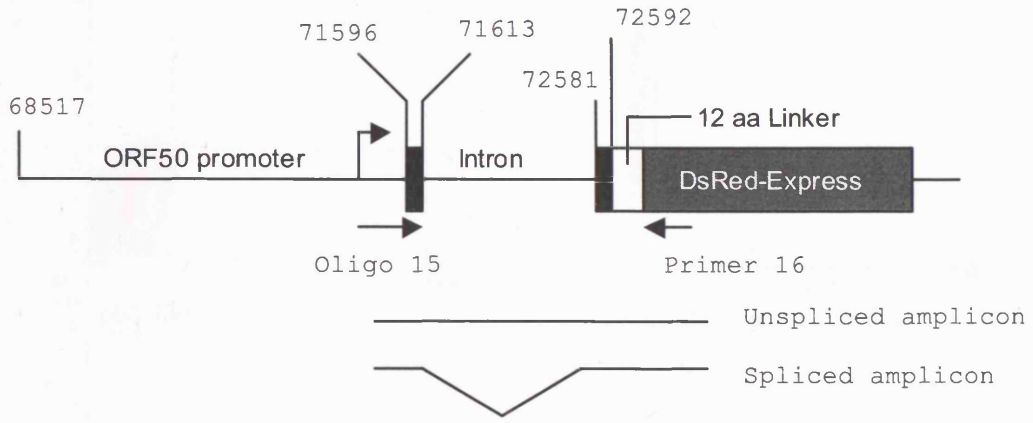
To investigate whether transfected XBP-1 can result in transcriptional activation of the ORF50 promoter, we generated the p50Redi reporter gene assay. Although many reporter gene assays have already been described which involve the ORF50 promoter, none have been adapted for use by flow-cytometry and not all contain the ORF50 intron.

Sequence analysis of the ORF50 promoter identified a putative XBP-1 binding site (Clauss *et al.*, 1996) within the ORF50 promoter between nucleotides 71826 and 71840 of the KSHV genome (Accession: NC_003409) depicted in figure 6.4.2. We therefore wanted to develop a reporter gene assay which maintained the intronic sequence and investigate whether the above sequence was an intronic enhancer element. Because our XBP-1 expression vectors also encode EmGFP we wanted to develop a flow-cytometry assay through which transfection efficiency could be monitored and 'double positive'-cells quantified.

The ORF50 promoter, first exon, intron and the first 7 codons of the second exon were PCR amplified and inserted into pCMV-DsRed-Express (Clontech) in place of the HCMV immediate early promoter generating p50Redi. The DsRed-Express protein is a human codon optimised version of DsRed with increased solubility and reduced green fluorescence (Bevis and Glick, 2002). The reduced green fluorescence means that DsRed-Express fluorescence can easily be separated from EmGFP fluorescence by flow cytometry. A diagrammatic representation of the p50Redi reporter-cassette is shown in figure 6.4.1 A. The fusion protein between the N-terminus of RTA and DsRed-Express is from hereon referred to as RTA-Express.

To assess whether p50Redi (detailed in section 2.2.7) was transcriptionally activated by XBP-1 expression, HEK 293-T cells were cotransfected with p50Redi and twice the molar equivalent of pIE, pXBPIG, pXBPUIG and pCMV-RTA (figure 6.4.1 C and D).

A



B

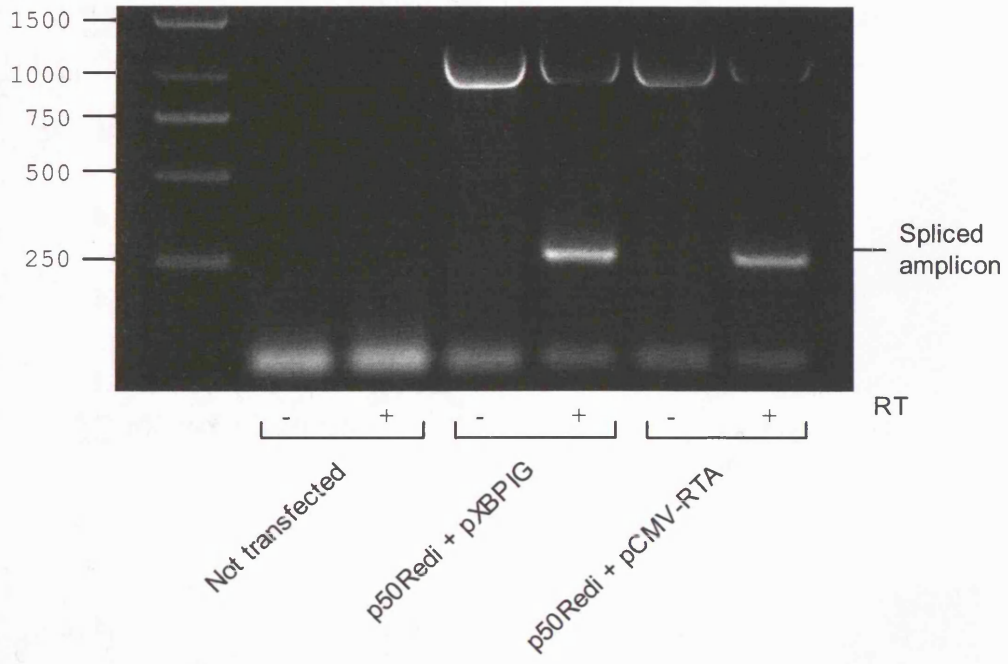


Figure 6.4.1. Activation p50Redi (continued overleaf)

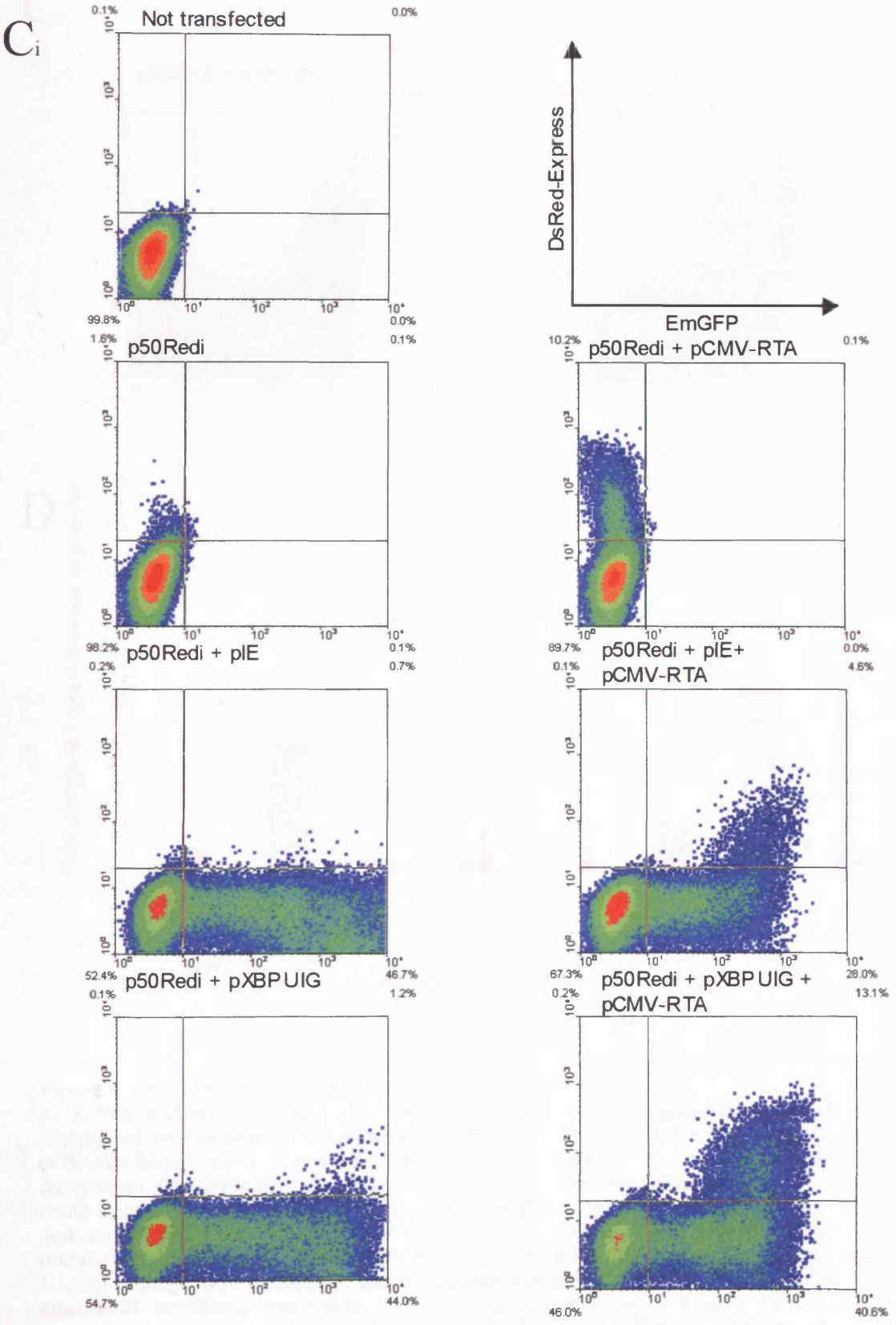


Figure 6.4.1. Activation of p50Redi (continued overleaf)

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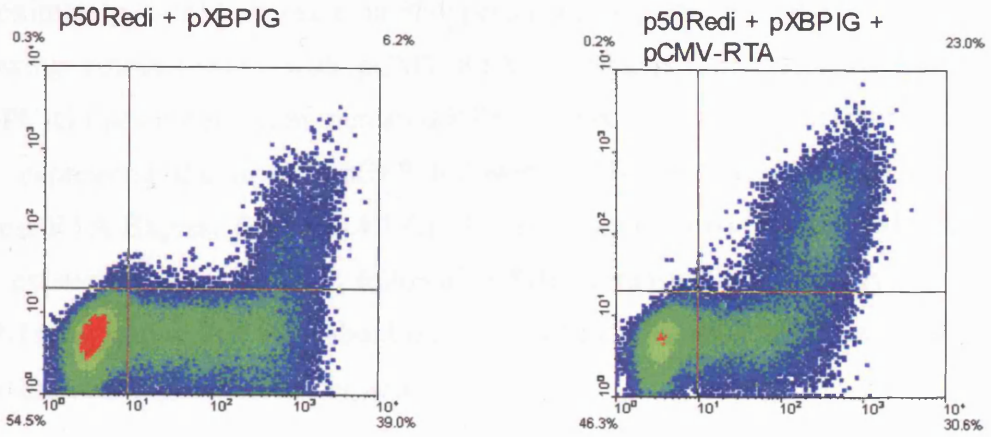

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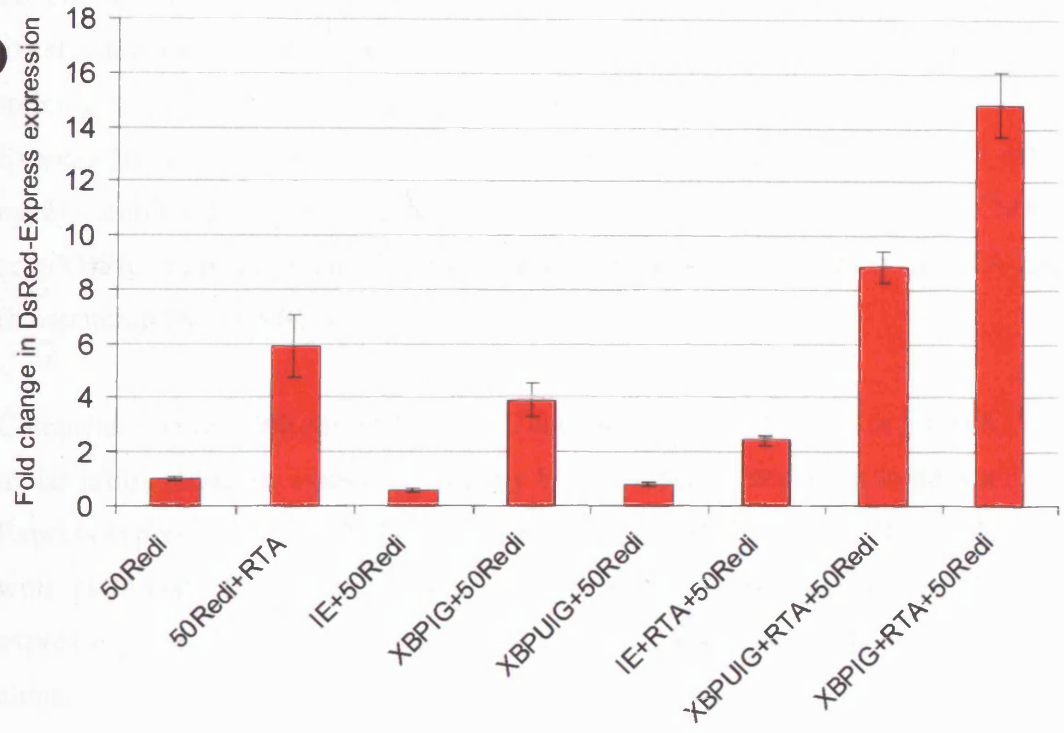


Figure 6.4.1. Activation of p50Redi
A. A diagrammatic representation of the RTA-Express reporter-cassette. Oligos 15 and 16 are highlighted as well as a 12 amino acid linker between RTA and DsRed-Express encoded by the pCMV-DsRed-Express polylinker. Numbers correspond to the KSHV genomic sequence (Accession: NC_003409). B. PCR amplification across the splice junction of RTA-Express using cDNA synthesis reactions from 4x10^6 HEK 293-T cells, transfected with 1 µg of p50Redi and double the molar equivalent of pCMV-RTA or pXBPIG, with or without the addition of reverse transcriptase. C. 4x10^5 HEK 293-T cells were seeded in triplicate and transfected with 1 µg of p50Redi and double the molar equivalent of pIE, pXBPUIG, pXBPIG, pCMV-RTA or a mixture of the above and made up to 3.8 µg with pBluescript II KS+ (Stratagene). DNA concentrations are listed in table 2.3.3.1. Density plots of EmGFP and RTA-Express expression 48-hours post transfection are shown. D A graphical representation of the density plots in C, Error bars represent the standard error between triplicate experiments.

Cotransfection with pXBPIG and p50Redi resulted in an approximately 4-fold increase in the percentage of cells expressing RTA-Express. This increase is similar to the approximately 6-fold increase in the percentage of cells expressing RTA-Express following cotransfection with pCMV-RTA. Crucially, cotransfection with pIE or pXBPUIG does not result in increased RTA-express expression. Interestingly, only the cells expressing the most EmGFP following pXBPIG and p50Redi cotransfection express RTA-Express (figure 6.4.1 C). Because EmGFP expression is IRES-mediated, cells expressing more EmGFP, following pXBPIG transfection, probably express more XBP-1s suggesting that a threshold level of XBP-1s expression has to be attained before RTA-Express expression can be activated.

To confirm that stimulation of the p50Redi ORF50 promoter, following pXBPIG transfection and pCMV-RTA transfection, results in faithful transcription initiation and splicing we carried out RT-PCR amplification across the splice junction of RTA-Express (figure 6.4.1 B). A 297 bp amplicon specifically amplified from cDNA is readily amplified from HEK 293-T cells cotransfected with p50Redi and pCMV-RTA or pXBPIG indicating faithful transcription initiation and splicing of RTA-Express transcription from p50Redi.

Cotransfection of p50Redi, pCMV-RTA and either pIE, pXBPUIG or pXBPIG in 1:1:1 molar ratios indicates a possible synergy between XBP-1 and RTA in activating RTA-Express expression (figure 6.4.1 C and D). Both pXBPUIG and pXBPIG cotransfection with pCMV-RTA and p50Redi results in a greater percentage of RTA-Express expressing cells than the molar equivalent of pXBPIG, pXBPUIG or pCMV-RTA alone.

6.4.2. Mutation of a putative XBP-1 binding site

To investigate whether the putative XBP-1 binding site between nucleotides 71826 and 71840 of the KSHV genome (Accession: NC_003409) is involved in pXBPIG activation of RTA-Express expression we mutated the sequence, in the context of p50Redi, generating pMut50Redi (figure 6.4.2 B). Transfection of pXBPIG, using the conditions described in section 6.4.1, activated RTA-Express expression by approximately 5-fold from pMut50Redi suggesting this sequence is unimportant in activating RTA-Express expression from p50Redi.

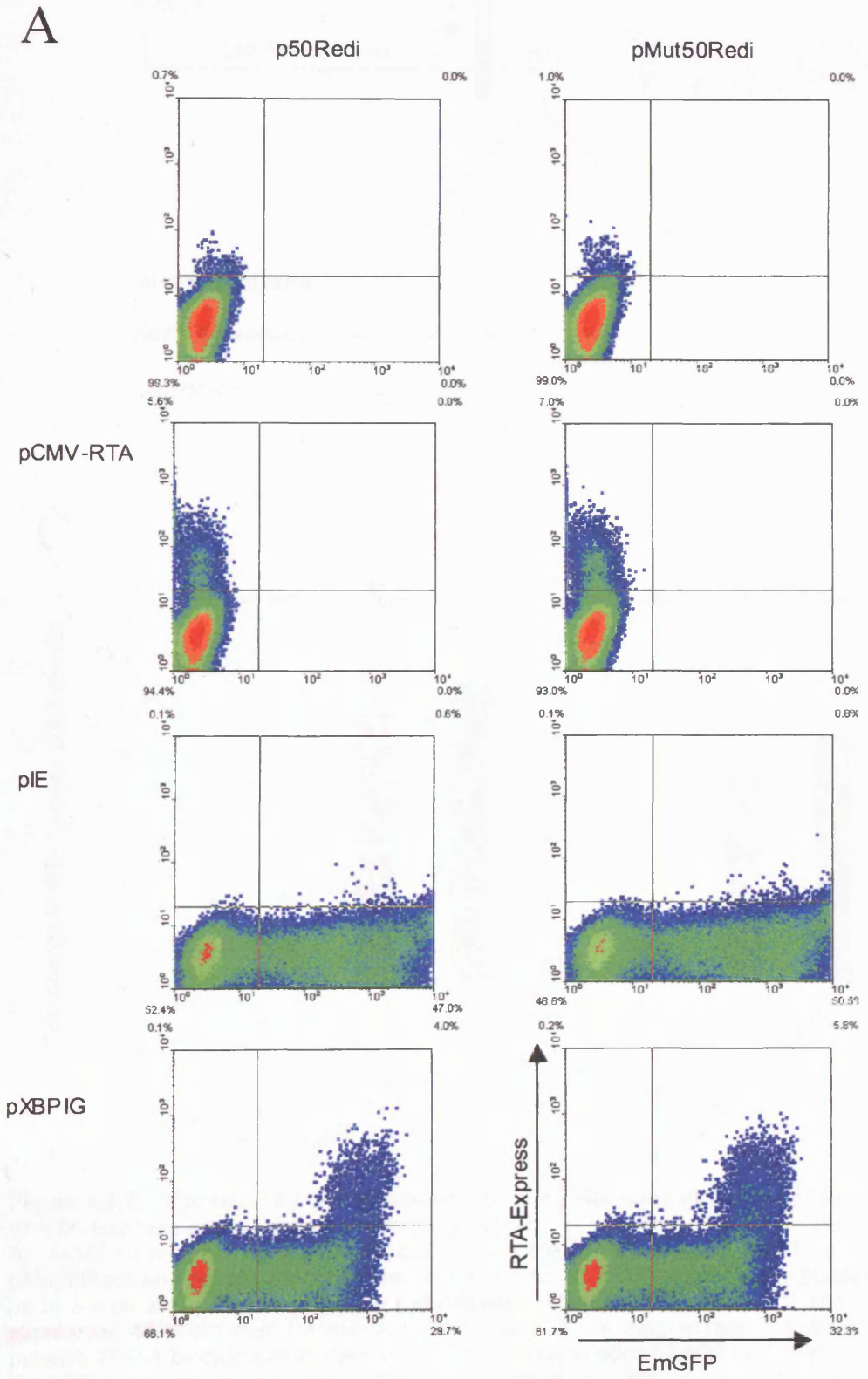
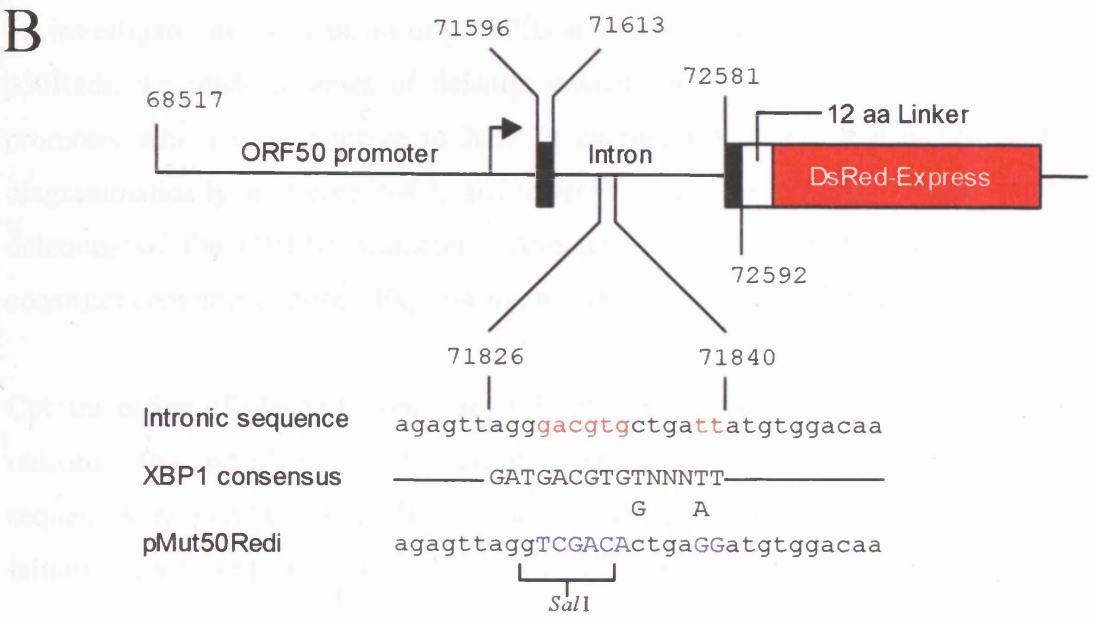


Figure 6.4.2. Mutation of a putative XBP-1 binding site does not affect pXBPIG activation of RTA-Express expression (continued overleaf).



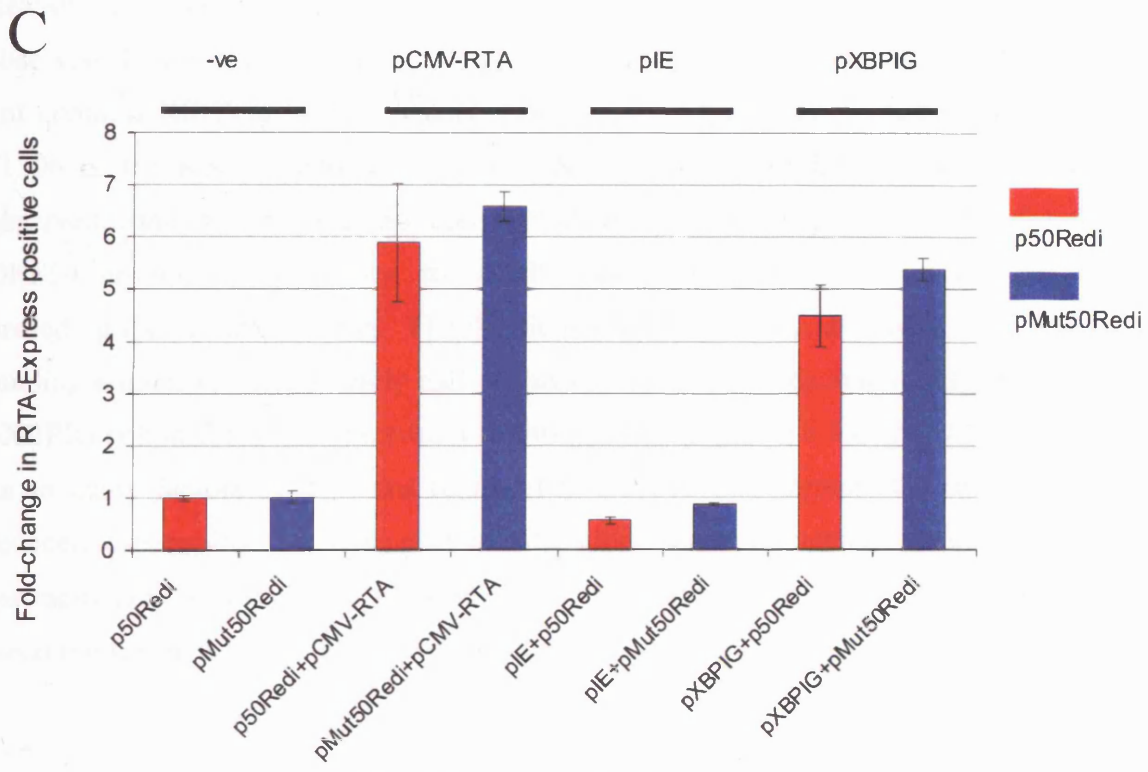


Figure 6.4.2. Mutation of a putative XBP-1 binding site does not affect pXBPIG activation of RTA-Express expression (continued overleaf).
A. 4x10^5 HEK 293-T cells were seeded in triplicate and transfected with 1 µg of p50Redi or pMut50Redi and double the molar equivalent of pIE, pXBPUIG, pXBPIG or pCMV-RTA, made up to 3.8 µg with pBluescript II KS+ (Stratagene). Density plots of EmGFP and RTA-Express expression 48-hours post transfection are shown. B. A diagrammatic representation of the putative XBP-1 binding site in the ORF50 intron. Nucleotides highlighted in red are identical to the XBP-1 consensus sequence defined in (Clauss *et al.*, 1996); nucleotides highlighted in blue are mutated in pMut50Redi. The highlighted *Sal* I restriction site was used for screening. C. A graphical representation of the density plots in A. Error bars represent the standard error between triplicate repeats.

6.4.3. Deletion analysis of the ORF50 promoter

To investigate the mechanism of pXBPIG activation of RTA-Express expression from p50Redi, we made a series of deletion mutants to define the region of the ORF50 promoter which is responsive to XBP-1s expression. These 9 plasmids, displayed diagrammatically in figure 6.4.3, are referred to as pΔ1760-pΔ3078 and contain 5' deletions of the ORF50 promoter. Another plasmid pNoRedi is a 'promoterless' construct containing DsRed-Express and no KSHV-derived sequence.

Cotransfection of plasmids containing ORF50 promoter deletions with pIE or pXBPIG indicates that pΔ2878 retains maximal promoter activity. This suggests that the sequences responding to pXBPIG transfection reside in the 200 bp preceding the initiation codon (71396-71596 of the KSHV genome, accession: NC_003409).

Reassuringly, minimal responsivity of pΔ3078 to cotransfection with pXBPIG was observed (Figure 6.4.3 C and D). This plasmid contains no ORF50 promoter sequence, but contains KSHV-derived sequence downstream of the initiation codon (nucleotide 71596 of the KSHV genome, accession: NC-003409). Similarly, no response was observed using the 'promoterless' construct pNoRedi, supporting the hypothesis that the ORF50 promoter responsivity, to pXBPIG cotransfection, resides in the 200 bp preceding the initiation codon. The deletion-construct pΔ2962, deleted 3' of the AP1-binding sequence, deletes nearly half of this region. A sharp decline in responsivity to pXBPIG cotransfection is observed with this deletion (Figure 6.4.3 C and D). However, as so many factors bind in this region it is difficult to determine the cause of this reduced responsivity. As some responsivity is maintained the reduction could be due to less activation in response to pXBPIG transfection or due to reduced coactivation from basal transcripion factors binding in this region.

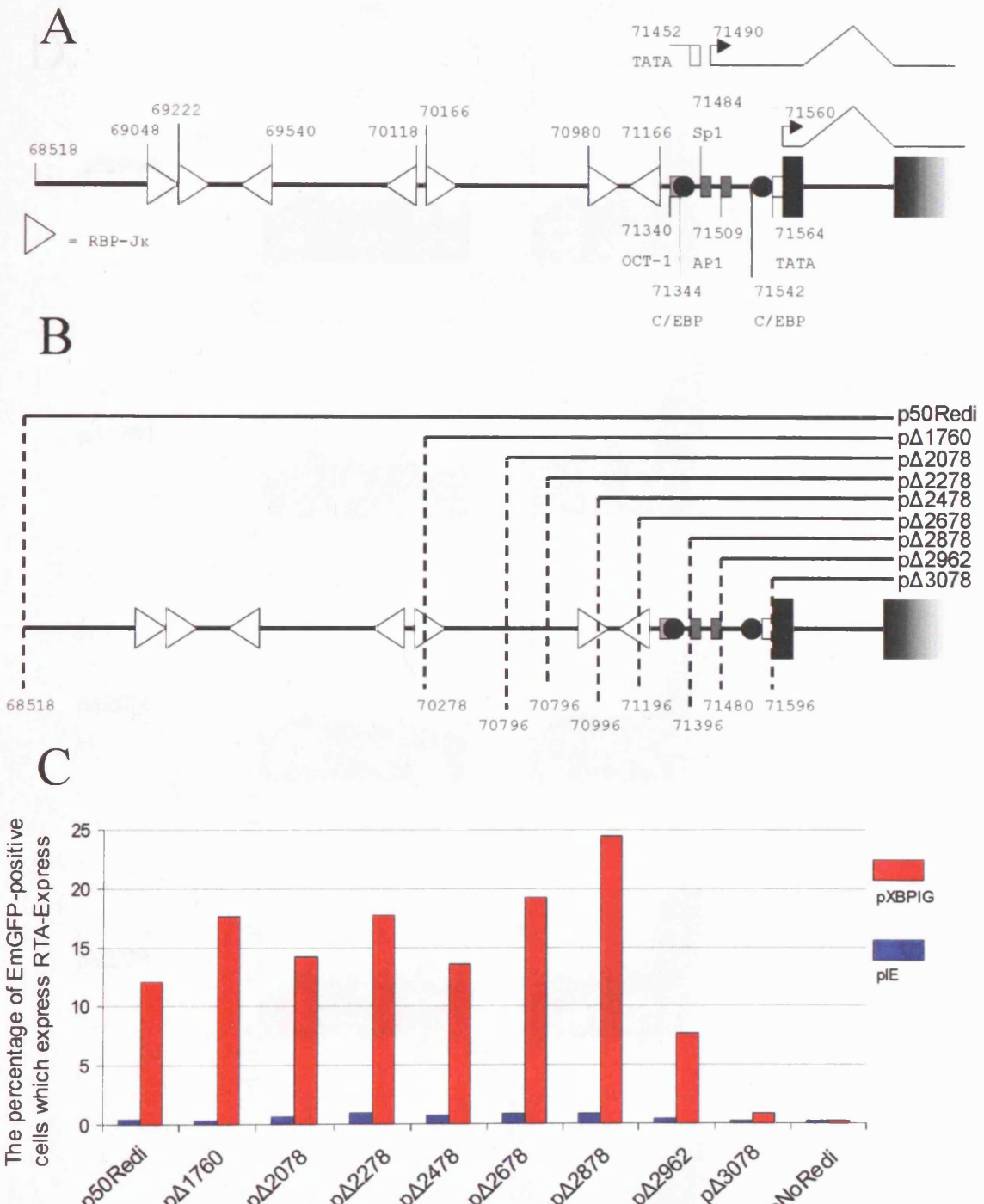


Figure 6.4.3. Deletion analysis of the ORF50 promoter (continued overleaf)

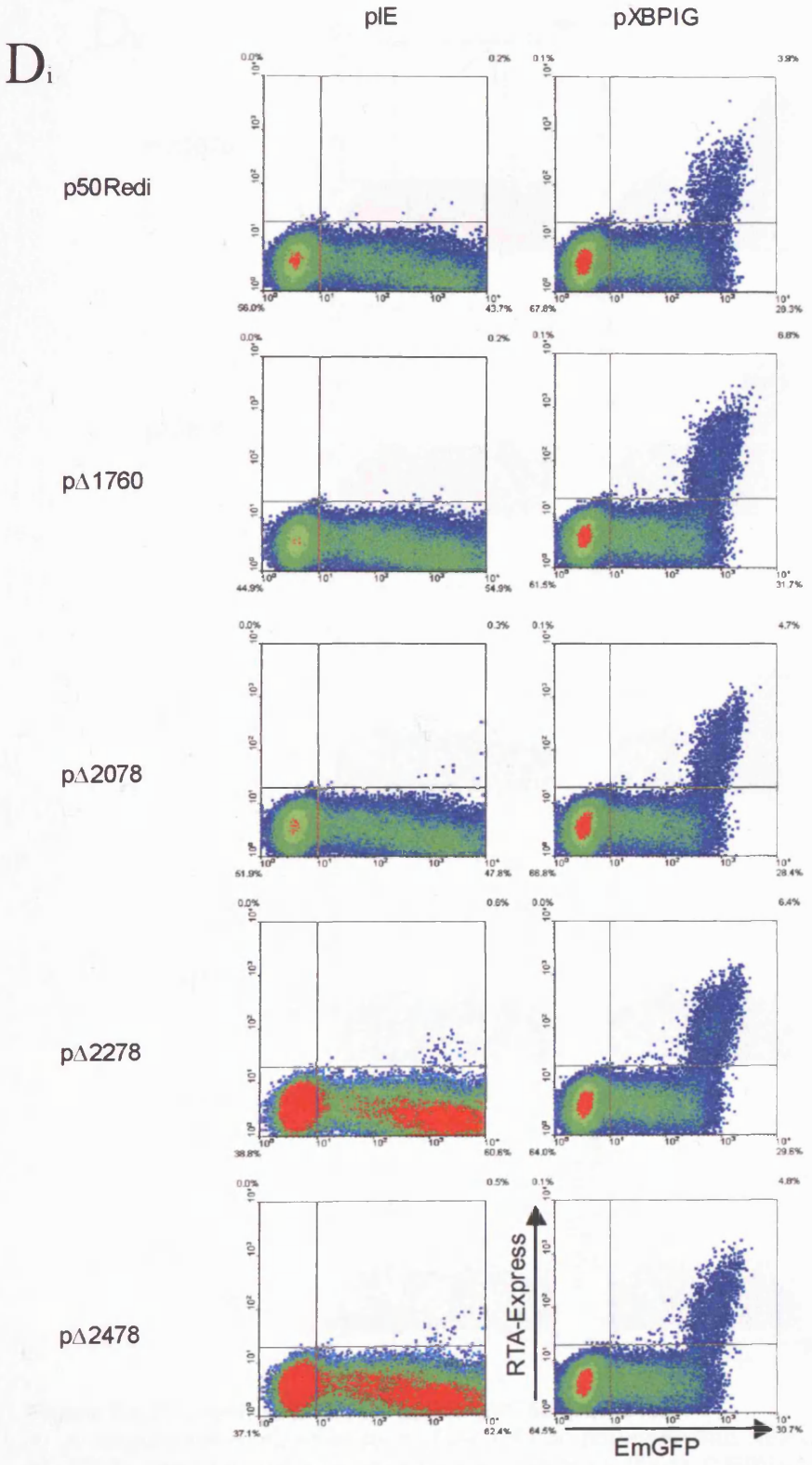


Figure 6.4.3. Deletion analysis of the ORF50 promoter (continued overleaf)

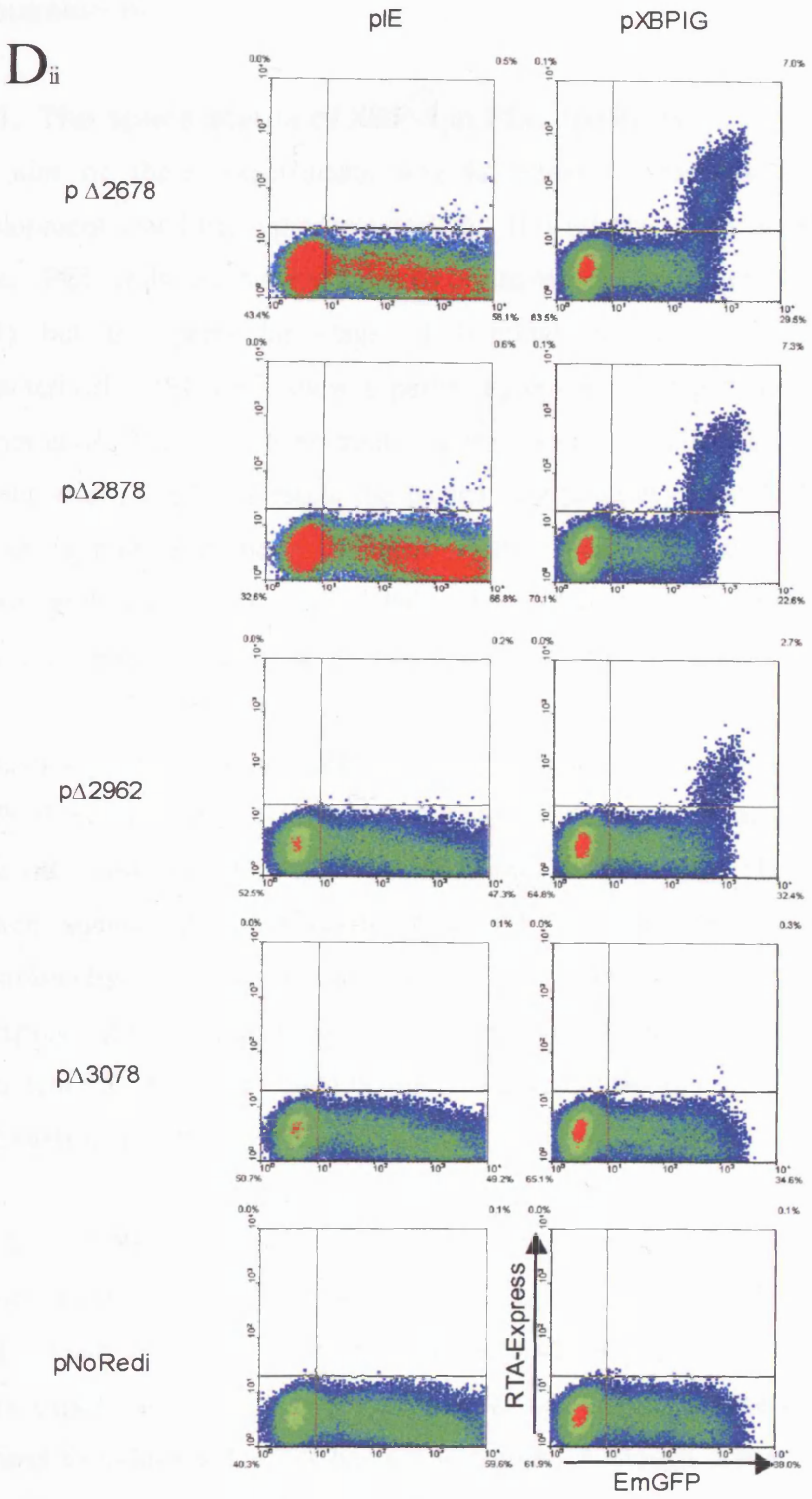


Figure 6.4.3. Deletion analysis of the ORF50 promoter.
A. A diagrammatic representation of the ORF50 promoter. The RBP-Jκ binding sites (Liang *et al.*, 2003), Oct-1 binding sequence (Sakakibara *et al.*, 2001), C/EBPα binding sites (Wang *et al.*, 2003), SP1 binding site (Ye *et al.*, 2005) and AP-1 binding site (Wang *et al.*, 2004b) are shown. The two reported TATA boxes and transcription initiation sites at 71560 (Lukac *et al.*, 1999) and 71490 (Deng *et al.*, 2000) are also highlighted. B. A diagrammatic representation of deleted ORF50 promoter used in pΔ1760-pΔ3078. C. $4x10^5$ HEK 293-T cells were seeded in triplicate and cotransfected with 1 µg of p50Redi, pNoRedi or a plasmid containing a 5' deleted ORF50 promoter (displayed in B) in addition to 2.8 µg of pXBPIG or 2.5 µg of pIE and 0.3 µg of pBluescript II KS+ (Stratagene). DNA concentrations are listed in table2.3.3.2. D. Representative density plots of the data in C.

Discussion IV

6.5.1. The splice-status of XBP-1 in PEL cell lines

The aim of these experiments was to further characterise the stage of B-cell development that PEL represents and how this relates to the control of the viral lytic cycle. PEL-cells are most similar to plasmablasts (Jenner *et al.*, 2003; Klein *et al.*, 2003) but the particular stage of terminal differentiation has not been fully characterised. PEL cells show a partial upregulation of genes expressed in the UPR (Jenner *et al.*, 2003) and consistent with this express the cleaved 50 kDa form of ATF-6 (Jenner *et al.*, 2003). Despite the partial upregulation of the UPR, PEL cells do not secrete immunoglobulin. Indeed, many PEL cells do not express surface immunoglobulin (Nador *et al.*, 1996). To further characterise the phenotype of PEL cells, we examined the splice status of XBP-1 in PEL.

Interestingly, PEL express XBP-1u but levels of XBP-1s are barely detectable by our RT-PCR assay (figure 6.1.2). In addition, it is clear that the machinery required to sense ER-stress and splice XBP-1 is functional in PEL cells. This is highlighted by the efficient generation of XBP-1s following DTT treatment and induction of an artificial ER-stress (figure 6.1.3). Taken together, these data suggest that PEL cells are capable of responding to ER-stress, but do not express a protein load required to generate a BiP dependent ER-stress. It would be interesting to corroborate these data with similar RT-PCR analysis of primary PEL cells.

The lack of XBP-1s expression is difficult to reconcile with the efficient cleavage of ATF-6 in these cells (Jenner *et al.*, 2003). The processing of ATF-6 in the absence of IRE1 activation suggests that PEL cells represent a stage of plasma cell differentiation where expansion of the secretory apparatus has begun, but the increased protein load required to induce ER-stress has yet to arrive. Although apparently at odds with our current understanding of the UPR, this sequence of events has been described by Heck and colleagues (van Anken *et al.*, 2003). In addition, ATF-6 is capable of being activated in a BiP independent fashion (Hong *et al.*, 2004a) suggesting that selective activation of the UPR may be normal cellular process.

In light of these observations, it is tempting to speculate that an inability to upregulate immunoglobulin expression may be the 'missing signal' preventing terminal

differentiation of PEL cells; 'trapping' them in a proliferating, plasmablastic state. Indeed, immunoglobulin secretion is never reported in PEL cells and many PELs do not express detectable levels of immunoglobulin (Nador *et al.*, 1996; Matolcsy *et al.*, 1998). In addition, PU.1 expression is not detectable in PEL cell lines (Arguello *et al.*, 2003). IRF4 heterodimerises with PU.1 and binds to enhancers of the kappa and lambda light chains (Eisenbeis *et al.*, 1995). The absence of PU.1 potentially reduces IgL expression in PEL cells.

6.5.2. Overexpression of XBP-1 in PEL

The paucity of XBP-1s expression in PEL cell lines led us to consider that plasma cell differentiation and XBP-1s expression might initiate KSHV reactivation from latency. With this in mind, we examined the affect of the XBP-1u expression vector XBPUIG and the XBP-1s expression vector XBPIG on KSHV reactivation. Unfortunately, the data in section 6.3 is not presented in chronological order. Because of time constraints, generating a sequence validated XBPUIG clone took substantially longer than generating an XBPIG clone. XBPUIG was therefore not included in the experiments described in figures 6.2.3 and 6.2.4. In addition, a 52 kDa protein is detected exclusively in XBPIG-transduced PEL cells (B. Webb, personal communication).

We are confident that XBP-1s is expressed following transduction of XBPIG. 48-hours post-transduction, XBPIG transduced cells have increased granularity. This increase is attributed to an expansion of the secretory apparatus and is similar to the phenotype described Staudt and colleagues (Shaffer *et al.*, 2004). Furthermore, XBP-1s transcript is readily detectable in XBPIG-transduced cells, but is largely absent from XBPUIG and IE-transduced controls (figure 6.2.2).

Interestingly, XBP-1s expression in PEL cells activates the KSHV lytic cycle. XBPIG-transduction of JSC-1, BCP-1 and BC-3 cell lines results in potent activation of RTA expression, an affect specific to transduced cells (as visualised by EmGFP expression). These three cell lines were chosen because they represent a broad spectrum of PEL cell lines encompassing EBV and KSHV dually infected JSC-1 cells (Cannon *et al.*, 2000), EBV negative BCP-1 and BC-3 cells (Boshoff *et al.*, 1998b; Arvanitakis *et al.*, 1996) and BC-3 cells, which lack the point mutations indicative of SHM suggesting an extra-GC pathway of maturation (Matolcsy *et al.*, 1998). Overexpressed XBP-1s is a potent activator of RTA-Expression in all three cell lines.

To investigate whether XBP-1s expression activates the entire lytic cycle, we examined the ability of transfected pXBPIG to influence recombinant KSHV production from clone 6 cells (described in chapter 5). Transfection of clone 6 cells with pXBPIG resulted in a greater than 6-fold increase is virus production (figure 6.2.4). This observation suggests that XBP-1s can activate the entire KSHV lytic cycle, presumably through upregulating RTA expression. Similarly, gene expression profiling demonstrates that many lytic genes are turned on following transduction with XBPIG (6.3.1 and 6.3.2). Taken together these data indicate that overexpressed XBP-1s is a potent inducer of KSHV lytic replication.

Somewhat surprisingly, microarray analysis suggested that XBPUIG also activates the KSHV lytic cycle. In the absence of endogenous XBP-1u protein expression data in PEL cell lines, it is difficult to interpret this observation. Although it is clear that overexpressed XBP-1u activates the KSHV lytic cycle, whether endogenous XBP-1u protein is expressed in PEL cell lines is an open question. However, rapid turnover of XBP-1u has been proposed as a major regulatory mechanism of XBP-1 target gene expression (Tirosh *et al.*, 2005b), and exogenous expression of XBP-1u may perturb this delicate balance. It is likely that the different gene expression profiles indicated in figures 6.3.1 and 6.3.2 represent different levels of lytic cycle induction. Comparing figures 6.3.2 and 6.2.3 suggests that fewer than 10% of cells express RTA following XBPUIG-transduction. In light of the microarray analysis described in figures 6.3.1 and 6.3.2 it would be interesting to repeat the experiments illustrated in figures 6.2.3 and 6.2.4, to quantify the relative ability of XBPIG and XBPUIG to activate KSHV RTA expression and recombinant virus production.

However, despite the unexpected activity of overexpressed XBP-1u, it is clear that XBPUIG-transduction does not result in the same magnitude of KSHV lytic gene expression as TPA-treatment or XBPIG-transduction of JSC-1 cells. To further characterise the roles of XBP-1u and XBP-1s it would be interesting to investigate the ability of endogenous XBP-1s to activate KSHV RTA expression in response to ER-stress.

6.5.3. p50Redi reporter gene assays

The aim of developing the p50Redi ORF50 reporter gene assay was to characterise how XBP-1 expression upregulates RTA expression. The ORF50 promoter in p50Redi is responsive to RTA expression and XBP-1s expression (figure 6.4.1). Interestingly, in cells transfected with pXBPIG, only cells expressing substantial levels of EmGFP are more likely to coexpress RTA-Express. Because EmGFP and XBP-1s are expressed from the same transcript, in pXBPIG-transfected cells, this suggests that a threshold of XBP-1s expression has to be attained before RTA-Express expression is activated. We are unable to directly compare the activation of RTA-Express expression by RTA and XBP-1s because they are expressed from different promoters in unrelated plasmid backgrounds.

Mutation of the putative XBP-1 binding site between nucleotides 71826 and 71840 of the KSHV genome (Accession: NC_003409) did not decrease the activation of RTA-Express expression mediated by XBP-1s (figure 6.4.2). In addition, XBP-1s is unable to activate RTA-Express expression from the pΔ3078 deletion of p50Redi. Taken together these data indicate that direct transactivation of RTA-Express expression, through XBP-1 binding to intronic sequences, in p50Redi, does not occur. To address which region of the RTA promoter responds to XBP-1s expression, we carried out deletion analysis of the p50Redi reporter plasmid. Interestingly, the 200 bp fragment of the ORF50 promoter, contained in pΔ2878, retained full responsivity to XBP-1s expression. It would be interesting to investigate whether XBP-1s and XBP-1u are capable of binding this DNA sequence directly. Understanding whether XBP-1 directly transactivates the ORF50 promoter or whether activation results from indirect interactions or downstream effects of XBP-1 over expression is central to understanding the role of XBP-1 in KSHV reactivation.

The synergistic activation of RTA-Express expression following cotransfection of pCMV-RTA with either pXBPUIG or pXBPIG is an interesting observation. Crucially, pXBPUIG transfection does not activate RTA-Express expression in the absence of pCMV-RTA (figure 6.4.1). However, cotransfection of pXBPUIG and pCMV-RTA resulted in greater activation of RTA-Express expression than the molar equivalents of pCMV-RTA or pXBPIG. In addition, this synergy is enhanced by cotransfection with pXBPIG in place of pXBPUIG. It would be interesting to investigate whether XBP-1

and RTA interact with each other and whether such an interaction is involved in their synergistic activation of RTA-Express expression.

It is tempting to speculate that the ability of over expressed XBP-1u to synergise with RTA in activating the ORF50-promoter partially explains the observation that XBPUIG transduction weakly activates KSHV lytic gene expression. Although over expressed XBP-1u, alone, seems unable to activate the ORF50 promoter in transient transfection assays, in the context of the latently infected PEL cell XBP-1u overexpression activates viral lytic gene expression. The ability of XBP-1u to synergise with RTA may account for the increased viral lytic gene expression in these cells. However, it is difficult to reconcile this explanation with the supposed presence of endogenous XBP-1u in PEL.

In summary, PEL cell lines seem to be arrested at a stage of B-cell development where the UPR is partially activated, containing processed ATF-6 (Jenner *et al.*, 2003) but lacking substantial levels of XBP-1s. This is analogous to an intermediate stage of plasma cell differentiation identified by Heck and colleagues (van Anken *et al.*, 2003). We have shown that although PEL cells lack substantial amounts of XBP-1s, they retain the ability to splice XBP-1 in response to ER-stress.

Using reporter gene assays we have demonstrated that over expressed XBP-1s is capable of activating the ORF50 promoter. Furthermore, we have shown that overexpression of XBP-1s, in PEL cells, results in RTA expression and induction of multiple viral lytic genes. Although the experiments discussed in this chapter provide a tantalizing glimpse into the possible role of plasma cell differentiation, ER-stress and XBP-1 in KSHV reactivation; we are currently unsure how XBP-1 activates RTA expression. In addition, we are unable to resolve the respective roles of XBP-1u and XBP-1s in modulating RTA expression.

Summary

This thesis describes the generation of an RNA polymerase III promoter driven, shRNA expression system capable of being delivered using lentiviral vectors and mediating RNAi. This strategy proved to be effective at attenuating GFP expression in a variety of cell lines. The 'knock down' of GFP expression was shRNA specific and these modified lentiviral vectors are capable of reducing their target gene expression by approximately 80-90% in multiple cell lines. These vectors are also capable of attenuating dEGFP expression in KSHV infected PEL cell lines indicating their potential utility in the 'knock down' of KSHV open reading frames.

As proof of principle that these vectors could be used to specifically interfere with KSHV gene expression, we targeted the well-characterised ORF50 mRNA and investigated the affect of RTA 'knock down' on KSHV lytic replication. We were able to prevent RTA expression in approximately 80% of cells. Crucially, this reduction was not seen with the control shRNA-DsRed suggesting this is a specific 'knock down' of KSHV RTA. Consistent with our current understanding of KSHV lytic replication, we observed a reduction in subsequent viral gene expression and virion production in cells expressing shRNA-50E. The potent specific interference with KSHV ORF50 expression described in chapter 4 suggests that these vectors could be used to target less defined KSHV genes, and assist in their characterisation.

Because RTA expression is necessary and sufficient for induction of KSHV lytic replication, we wished to examine host-cell triggers for its initial expression. With this in mind, we examined the XBP-1 splice-status of PEL cell lines and investigated the affect of XBP-1 overexpression on KSHV lytic reactivation. Interestingly PEL cells have a partially activated UPR. ATF6 is processed in PEL cell lines (Jenner *et al.*, 2003), but XBP-1s expression is largely absent in PEL cell lines. Despite the paucity of XBP-1s in PEL cell lines, PEL cells retain the ability to splice XBP-1 in response to a chemically induced UPR, suggesting that PEL cells are lacking sufficient ER-stress to generate XBP-1s. In a developmental context this suggests that PEL cells represent plasmablasts arrested close to the plasma cell fate, lacking the physiological protein load required to splice XBP-1.

Overexpressed XBP-1 is capable of activating the ORF50 promoter in transient transfection, reporter gene assays. When overexpressed in the context of latently infected PEL cells, XBP-1s efficiently induces RTA expression and subsequent initiation of the KSHV lytic cycle. Both overexpressed XBP-1s and XBP-1 u are capable of synergising with RTA in the activation of the ORF50 promoter in transient reporter gene assays. This could partially explain the KSHV lytic gene expression observed in PEL cells overexpressing XBP-1u. The experiments described highlight a possible role for XBP-1 and plasma cell differentiation in initiating KSHV reactivation. F`uture experiments must examine the ability of endogenous XBP-1 to activate the KSHV lytic cycle, define whether XBP-1 binds the ORF50 promoter directly and investigate the synergistic action of RTA and XBP-1.

Chapter 7
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Appendix

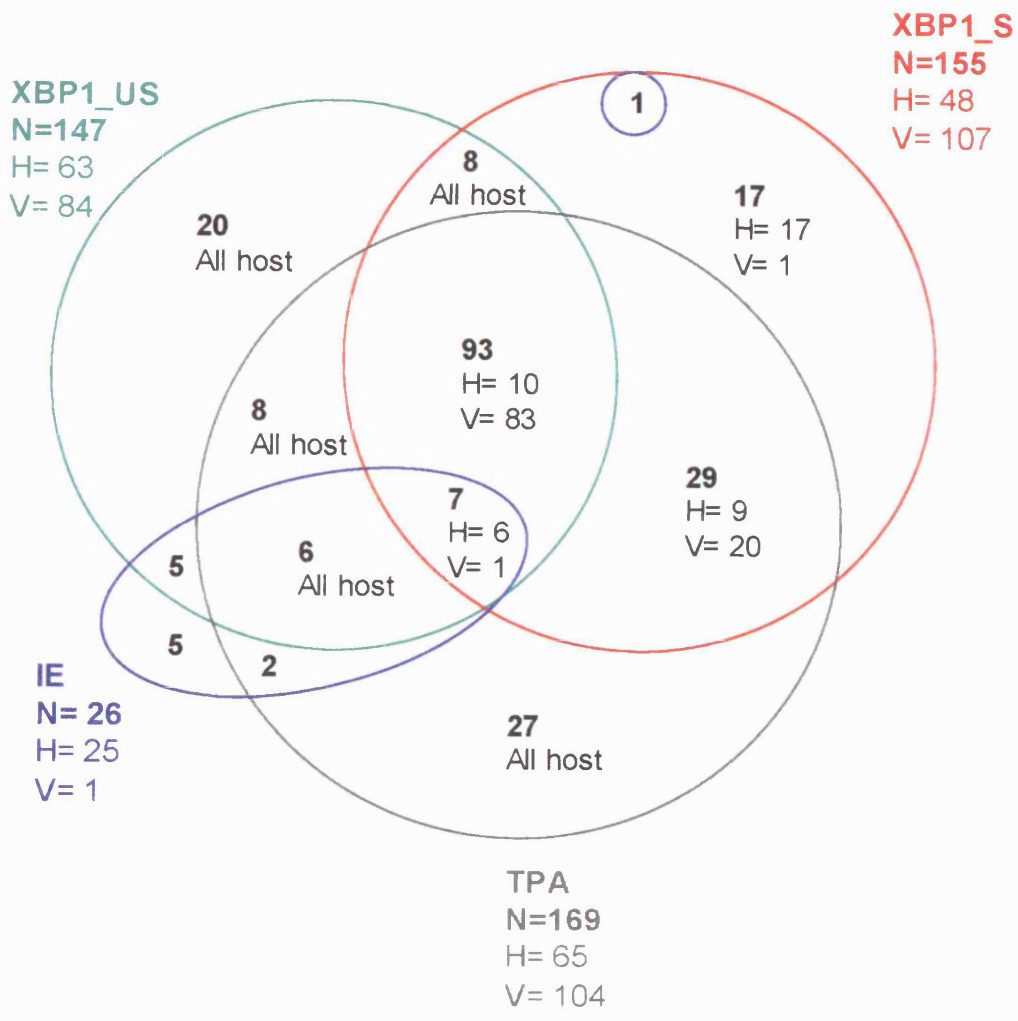


Figure 1.5. KSHV and host core and diverging transcriptional upregulation in JSC-1, in response to TPA, IE, XBP-1s and XBP-1us.
Venn diagram showing numbers of significantly up regulated KSHV and host genes, as compared to in normal JSC-1s, in JSC-1s stimulated with TPA, IE, XBP-1s and XBP-1us.

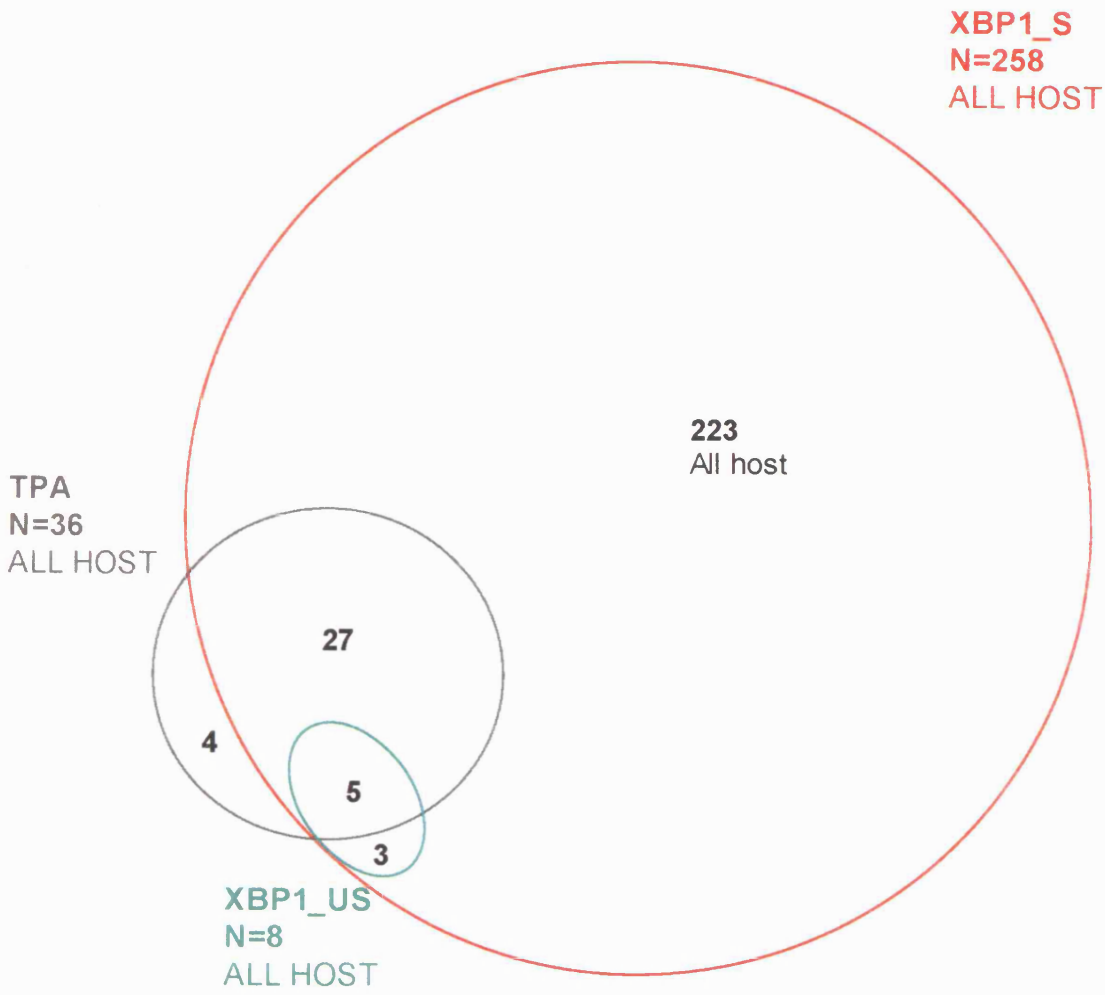
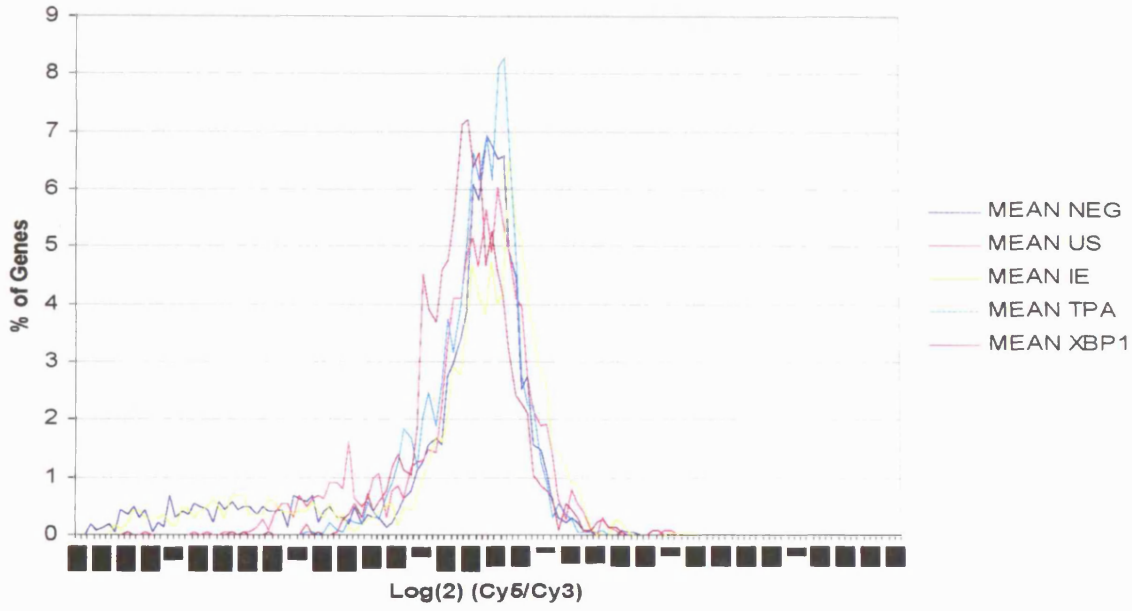


Figure 1.5. Host core and diverging transcriptional downregulation in JSC-1, in response to TPA, IE, XBP-1s and XBP-1us.
Venn diagram showing numbers of significantly down regulated genes, as compared to normal JSC-1s, in JSC-1s stimulated with TPA, IE, XBP-1s and XBP-1us. Only host genes are down-regulated.



	JSC-1 (-)	XBP1_U	IE	TPA	XBP1_S
Mean	-0.6388348	-0.2594841	-0.4198685	-0.0728714	-0.2393909
Standard Deviation	1.66828131	1.09419924	1.7049816	0.66453549	0.73617772
Sample Variance	2.78316254	1.19727197	2.90696226	0.44160742	0.54195763
Range	8.8518701	8.45385431	9.35550155	4.83521006	8.65444353

Figure 4.9 Evaluation of variation in gene expression data across all experimental conditions.
(A) Graph represents the distribution of mean log(2) ratios for each array element (after filtering) for JSC-1 cells stimulated with TPA, XBP-1s, XBP-1us, IE and unstimulated JSC-1s. For the latter two experimental conditions the distribution is slightly skewed to the left, as in comparison to the other three experimental conditions these samples possessed significantly fewer upregulated host and viral genes. The other three distributions are comparable. This is important to establish as large variations in sample distribution and variance can affect the way that SAM computes significantly regulated genes: all response variables must be comparable. **(B)** Table of general statistics derived from the log(2) expression data for each array element (after filtering) for JSC-1 cells stimulated with TPA, XBP-1s, XBP-1us, IE and unstimulated JSC-1s. The sample distribution (standard deviation) and variance are higher for the two experimental control conditions (JSC-1neg and IE), compared to the most highly transcriptionally activated samples (TPA and XBP1s), as a result of convergent and focussed gene expression occurring during activation of KSHV lytic replication. XBP1us is intermediary between the two.